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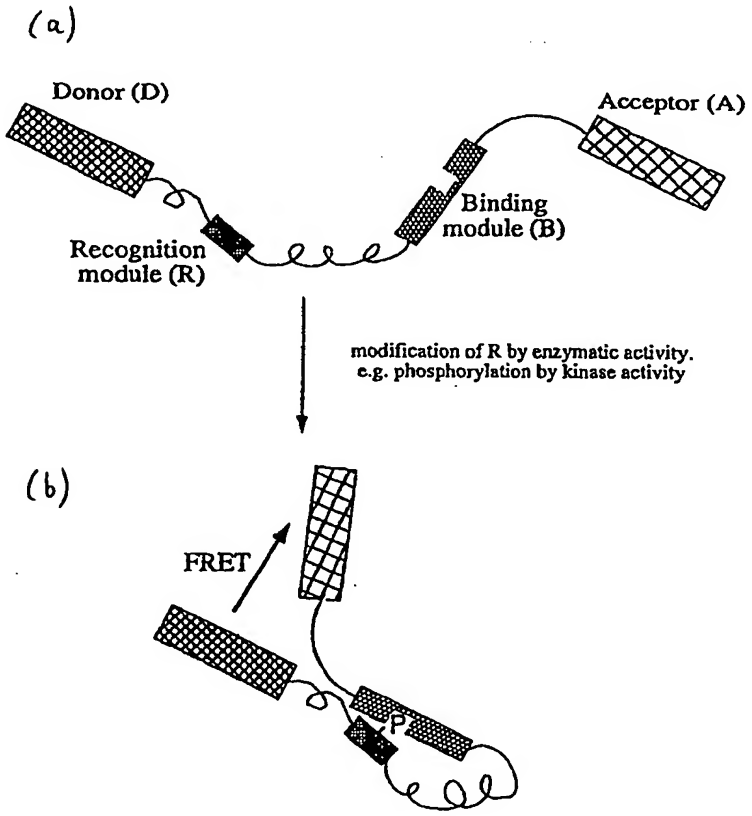
(54) Title: FLUORESCENT ASSAY FOR BIOLOGICAL SYSTEMS

(57) Abstract

A method for the measurement of a degree of fluorescence resonance energy transfer taking place between a donor and acceptor system by the steps of: irradiating a combined donor–acceptor system with a beam of intensity modulated excitation energy of a first wavelength; receiving fluorescence emissions from the donor and acceptor molecules having overlapping spectra; simultaneously determining a modulation lifetime (τ_{mod}) and a phase lifetime (τ_{ϕ}) of the combined emitted fluorescence of the donor–acceptor system; and determining a degree of acceptor ingrowth by comparison of τ_{mod} and τ_{ϕ}. The method is particularly suited to measurements of a degree of FRET in a biological system.



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FLUORESCENT ASSAY FOR BIOLOGICAL SYSTEMS

The present invention relates to a fluorescent assay for biological systems which uses biological reporter constructs and also to techniques for monitoring conformational changes in the constructs using fluorescence lifetime imaging.

Fluorescent assays for biological systems have been used for several years since, compared to many biological techniques, they have the advantage of, in some circumstances, being able to be carried out non-invasively and also they are able to give real time analysis of particular reactions in complex biological systems in which many reactions are being carried out simultaneously. In conjunction with the development of physical techniques for fluorescent assays has been the development of biological reporter constructs which serve as monitors of reactions, for example, within a cell. In particular, the development of fluorescent proteins that do not require cofactors for their intrinsic fluorescence has meant that such proteins can be introduced into and expressed in cells *via* genetic constructs.

Examples of biological reporters for fluorescent assays, and details of intrinsically fluorescent proteins, notably so-called "green fluorescent proteins" or "GFPs" (although they may be blue or yellow) are known. Miyawaki *et al* (1997) *Nature* **388**, 882-887 describes a GFP-based Ca^{2+} sensing system; Mitra *et al* (1996) *Gene* **173**, 13-17 describes a two-GFP-based system for use in identifying protease inhibitors; WO 97/28261 discloses a two-GFP system in which the GFP donor and GFP acceptor are linked by a peptide containing a protease cleavage site. WO 95/07463

describes uses of GFPs; WO 96/23898 relates to a method of detecting biologically active substances using GFPs; Heim & Tsien (1996) *Current Biology* **6**, 178-182 relates to engineered GFPs with improved brightness, longer wavelengths and fluorescence resonance energy transfer (FRET); Poppenborg *et al* (1997) *J. Biotechnol.* **58**, 79-88 relates to GFPs as a reporter for bioprocess monitoring; Park & Raines (1997) *Protein Science* **6**, 2344-2349 relates to a GFP as a signal for protein-protein interactions; Niswender *et al* (1995) *J. Microscopy* **180**, 109-116 relates to quantitative imaging of GFP in cultured cells; Chalfie *et al* (1994) *Science* **263**, 802-805 relates to GFP as a marker for gene expression; Hampton *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 828-833 relates to the *in vivo* examination of membrane protein localization and degradation with GFP; Heim *et al* (1995) *Nature* **373**, 663-664 relates to mutant GFPs with altered fluorescent properties; Mosser *et al* (1997) *BioTechniques* **22**, 150-161 relates to the use of a dicistronic expression cassette encoding GFP for the screening and selection of cells expressing inducible gene products; Suarez *et al* (1997) *Gene* **196**, 69-74 relates to GFP-based reporter systems for genetic analysis of bacteria; Niedenthal *et al* (1996) *Yeast* **12**, 773-778 relates to GFP as a marker for gene expression and subcellular localization in budding yeast; and Prescott *et al* (1997) *FEBS Lett* **411**, 97-101 relates to the use of GFP as a marker for assembled mitochondrial ATP synthase in yeast. GFPs and their uses have been reviewed in Pozzan *et al* (1997) *Nature* **388**, 8340-835, Misteli & Spector (1997) *Nature Biotechnology* **15**, 961-964; and Cubitt *et al* (1995) *Trends Biochem. Sci.* **20**, 448-455.

Reporter systems which are not based on GFPs but which may find use in biological systems are known, for example in Zlokarnik *et al* (1998) *Science* **279** 84-88 and Bastiaens & Jovin (1996) *Proc. Natl. Acad. Sci. USA* **93**, 8407-8412.

WO 97/08538 relates to fluorescence lifetime-based imaging and spectroscopy in tissues. WO 94/18547 relates to apparatus for quantitative imaging of multiple fluorophores. WO 92/13265 relates to method and apparatus for multi-dimensional phase fluorescent lifetime imaging. WO 92/07245 relates to method and apparatus for performing phase fluorescence lifetime measurements in flow cytometry.

A particular problem arises when the spectral separation of donor and acceptor fluorescence spectra are not well-resolved, and determination of fluorescence resonance energy transfer (FRET) within the donor-acceptor system cannot be determined by prior art methods such as using optical filtering. This is particularly problematic in relation to the use of GFPs and mutant GFPs.

It is therefore an object of the present invention to provide a method suitable for determining a degree of FRET in a donor-acceptor system not spectroscopically well-resolved, particularly for use in biological systems.

It is a further object of the invention to provide improved biological reporter constructs whether for use with the improved method of determining a degree of FRET in a donor-acceptor system of the present invention, or for use with prior art methods of determining a degree of FRET as said.

According to one aspect, the present invention provides a method for the measurement of a degree of fluorescence resonance energy transfer taking place between a donor and acceptor system by the steps of:

irradiating a combined donor–acceptor system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules having overlapping spectra;

simultaneously determining a modulation lifetime (τ_{mod}) and a phase lifetime (τ_{ϕ}) of the combined emitted fluorescence of the donor–acceptor system; and

determining a degree of acceptor ingrowth by comparison of τ_{mod} and τ_{ϕ}.

According to a further aspect, the present invention provides a method for the measurement of a degree of fluorescence resonance energy transfer taking place between a donor and acceptor system by the steps of:

irradiating a combined donor-acceptor system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules having overlapping spectra;

carrying out a Fourier transform on the modulated fluorescence emissions from the donor and acceptor system;

determining an amplitude of a time independent component, a time dependent cosine component and a time dependent sine component; and;

determining a measure of FRET efficiency from said components.

According to a further aspect, the invention provides a method for determining the relative state populations of a biological system capable of having two states, in which one state exhibits a first degree of FRET between donor and acceptor molecules and in which a second state exhibits

a second degree of FRET between donor and acceptor molecules, the method comprising the steps of:

irradiating the biological system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules of the first state and of the second state, the fluorescence emissions having overlapping spectra;

carrying out a Fourier transform on the modulated fluorescence emissions having overlapping spectra;

determining an amplitude of a time independent component, a time dependent cosine component and a time dependent sine component; and

determining the relative population of the first state and the second state therefrom.

According to a further aspect, the invention provides a method of detecting a change in a biological system from a first state to a second state wherein the biological system comprises a donor and acceptor system wherein in changing from the first state to the second state a change in the degree of fluorescence resonance energy transfer takes place between the donor and acceptor system, the method comprising the steps of:

irradiating the biological system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules having overlapping spectra;

simultaneously determining a modulation lifetime (τ_{mod}) and a phase lifetime (τ_{ϕ}) of the combined emitted fluorescence of the donor-acceptor system; and

determining a change in the degree of acceptor ingrowth by comparison of τ_{mod} and τ_{ϕ}.

The "biological system" may be any suitable biological system which contains a suitable donor and acceptor system. The biological system may be a cell which contains a suitable donor and acceptor system or it may be an *in vitro* biological system, for example one that contains a protein that constitutes a suitable donor and acceptor system and that can change from a first state to a second state.

Typically, in the first state the donor and acceptor are juxtaposed so that FRET is inefficient or does not occur to a substantial extent (at least in a proportion of the donor/acceptor systems within the biological system) whereas, typically, in the second state the donor and acceptor are juxtaposed so that FRET is more efficient than in the first state or occurs to an extent. It will be appreciated that the definitions of the first and second states could be reversed and that, for the method of the invention to be useful, there is a measurable difference in the degree of FRET which takes place between a donor and acceptor system when in the first state of the biological system and when in the second state of the biological system.

The biological systems which contain a suitable donor and acceptor system may be a prior art system such as that described in Miyawaki *et al* (1997) *Nature* **388**, 882-887 or it may be a new system as disclosed in detail below.

Conveniently, the donor and acceptor are comprised in a biological reporter construct. In a preferred embodiment the donor and acceptor are polypeptides which require no cofactor in order to be fluorescent (ie the polypeptides are intrinsically fluorescent once synthesised). Particularly preferred donors and acceptors include "green fluorescent proteins" (GFPs) which have suitable spectral characteristics in order to behave, as the case may be, as a donor or acceptor in a fluorescence resonance energy transfer (FRET) reaction. GFPs are produced naturally by *Aequorea victoria* but, as is well known in the art and described, for example, in Mitra *et al* (1996) *Gene* **173**, 13-17; Cubitt *et al* (1995) *Trends Biochem. Sci.* **20**, 448-454; Miyawaki *et al* (1997) *Nature* **388**, 882-887; Patterson *et al* (1997) *Biophys J.* **73**, 2782-2690; Heim & Tsien (1996) *Curr. Biol.* **6**, 178-182; and Heim *et al* (1995) *Nature* **373**, 663-664, mutant GFPs are available which have modified spectral characteristics. Certain GFPs and mutant GFPs are available from Clontech Laboratories UK Ltd, Wade Road, Basingstoke, Hants RG24 8NE. A particularly suitable mutant GFP is described in copending UK patent application entitled "Fluorescent Protein" and the PCT Patent Application which claims priority from that patent application and which was filed on the same day as this application.

The methods of the invention are particularly suited to investigating changes in biological systems either within a cell (for example, so that the effect of test compounds on a cell can be measured) or in an *in vitro* system. Both cell-based and *in vitro* systems have many uses, such as the identification of compounds which modulate a biological system in a specific way. It is envisaged that the methods of the invention will be particularly suited to screening assays for drugs that modulate a biological system in a desirable way, for example, by inhibiting an enzyme reaction

which inhibition can be detected by a change in the degree of FRET taking place between a donor and acceptor system in a biological system which contains a suitable donor/acceptor system.

Although, as noted above, the methods of the invention may be used with any suitable biological system, it is especially useful when used for detecting FRET in the biological system comprising a new donor/acceptor system as now described.

A further aspect of the invention provides a polypeptide comprising, in any order in the polypeptide chain, (1) a donor chromophore, (2) an acceptor chromophore, (3) a domain R comprising an enzyme recognition site and (4) a domain B which either (a) binds to R once the enzyme has acted on the said recognition site or (b) binds to R when the enzyme has not acted on the said recognition site but does not bind to R once the enzyme has acted on the said recognition site and when B is bound to R, and when appropriately irradiated, there is a change in the degree of fluorescence resonance energy transfer taking place between the donor and acceptor chromophore compared to when B is not bound to R.

It will be appreciated that the polypeptides of this aspect of the invention are useful in screening assays for agents that modulate enzyme activity.

A still further aspect of the invention provides a library of polypeptides, the polypeptides each comprising, in any order in the polypeptide chain, (1) a donor chromophore, (2) an acceptor chromophore, (3) a domain B for which it is desired that a polypeptide binding partner is identified, and (4) a domain R which may bind to domain B and wherein when R is bound

to B, and when appropriately irradiated, there is a change in the degree of fluorescence resonance energy transfer taking place between the donor and acceptor chromophore compared to when R is not bound to B, and wherein each member of the library has the same donor chromophore, acceptor chromophore and domain B, but different members of the library have different domains R.

It will be appreciated that the library of polypeptides of this aspect of the invention will contain a polypeptide wherein B binds to R. Thus, the library system may be used to identify one or more domains R that bind to domain B. It will be appreciated that the variable domain R may be a domain which is encoded by a polynucleotide which has been randomly selected, for example from a cDNA library (from a tissue in which a polypeptide is expected to exist which binds to domain B) or which has been randomly synthesised. In any case, the library of polypeptides is useful in screening for and identifying a binding partner for domain B from a plurality of possible such binding partners from a population of R domains.

A further aspect of the invention provides a polynucleotide encoding said polypeptides or, as the case may be, polynucleotides encoding the library of polypeptides. Typically, the polynucleotides is or is comprised in an expression vector.

A still further aspect of the invention provides a cell comprising a polynucleotide encoding a said polypeptide or an expression vector comprising such a polynucleotide. Still further aspects of the invention provide the use of the polypeptides or library of polypeptides or

polynucleotides or expression vectors or host cells comprising said polynucleotides in a screening assay.

The expression vectors, typically capable of expression in mammalian cells, encoding the polypeptides, consisting of the four modules, can be constructed by standard laboratory molecular biology methods such as those described in Sambrook *et al* (1989) *Molecular cloning, A laboratory manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York incorporated herein by reference.

The polynucleotide of the invention (typically DNA) may be expressed in a suitable host to produce a polypeptide of the invention. Thus, the DNA encoding the polypeptide of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The polynucleotide, such as DNA, encoding the polypeptide of the invention may be joined to a wide variety of other DNA sequences for introduction

into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted in to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are

then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and

YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cells, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is

consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

A suitable "starting" vector is the pcDNA3.1 vector distributed by Invitrogen (Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands). The key features of this vector for this invention are: (i) Cytomegalovirus enhancer-promoter for high level expression of the insert in mammalian cells (the insert is for example the cDNA encoding the polypeptides described above and has to be cloned into the vector); (ii) multiple cloning site in forward and reverse orientation; (iii) expression cassette for a selectable marker in eukaryotic cells (neomycin, zeocin or hygrommycin).

Typically, an entire construct may be engineered by the following steps.

1. Amplification of the cDNAs encoding the four individual modules and attached spacers by the polymerase chain reaction (PCR). The primers used for this must be partly compatible with the cDNAs amplified and partly compatible with the vector/coding sequence of the construct the amplification products are cloned into. Their sequence varies from case to case.

2. Sequential ligation of the individual amplification products into a suitable vector such as the pcDNA3.1 vector.

Embodiments of the present invention will now be described by way of example and with reference to the accompanying drawings in which:

Figure 1 shows a schematic diagram of a number of possible arrangements of donor-acceptor-recognition-binding molecules according to the present invention;

Figure 2 illustrates a possible conformational change in the construct of Figure 1(a) which can be measured by determining the degree of FRET between the two chromophores;

Figure 3 shows a schematic diagram of energy transfer in and between the donor and acceptor molecules;

Figure 4 shows a graph of fluorescence emission as a function of time, for varying degrees of FRET efficiency, in a donor-acceptor system according to the present invention, in which the donor alone has a fluorescence lifetime of 2 nanoseconds (ns) and the acceptor alone has a fluorescence lifetime of 4 ns;

Figure 5 shows fluorescence lifetimes of a donor-acceptor system according to the present invention as a function of FRET efficiency, and frequency in which: Figure 5(a) illustrates the modulation lifetime; Figure 5(b) illustrates the phase lifetime; and Figure 5(c) illustrates the ratio of modulation and phase lifetimes for a system in which the donor alone has a fluorescence lifetime of 2 nanoseconds (ns) and the acceptor alone has a fluorescence lifetime of 4 ns;

Figure 6 shows a parametric plot of phase lifetime *vs.* modulation lifetime as a function of the relative population (*f*) of cleaved construct for different FRET efficiencies for a construct which contains a protease cleavage site;

Figure 7 shows experimental fluorescence lifetime images and a 2D histogram of the phase and modulation lifetimes of Cos 7 cells expressing a construct which contains a caspase cleavage site. The images and the results in the 2D histogram are shown before and 2 hours after addition of the apoptosis inducing ligand Fas to the cells.

Figure 8 is a schematic representation of a construct (cDNA) for measuring protease activity by FRET;

Figure 9 is a schematic representation of a construct (cDNA) for measurement of cAMP;

Figure 10 is a schematic representation of a polypeptide for measuring extracellular insulin;

Figure 11 is a schematic representation of how to use SH2 domain-phosphotyrosine peptide interactions as kinase assays in the Jak Kinase/STAT pathway;

Figure 12 shows a schematic diagram of apparatus suitable for carrying out the method of the present invention;

Figure 13 shows a flowchart of the steps for carrying out single frequency FRET lifetime measurements; and

Figure 14 shows a flowchart of the steps for carrying out multi-frequency FRET lifetime measurements.

With reference to Figure 1, there is shown a single polypeptide consisting of four modules (or domains) linked by spacers which can be constructed and expressed in cells by standard recombinant DNA technology and transfection (or transformation) methods as described above. The first module or domain is a green fluorescent protein which acts as a donor chromophore D in an excited state energy transfer reaction. The second module (or domain) is a distinct green fluorescent protein with a red-shifted spectrum compared to the donor D which acts as an acceptor chromophore A in the excited state energy transfer reaction. The third module R is an enzyme specific recognition site. The fourth module B is a protein domain which specifically binds to, or is released from the R module upon enzymatic modification of it. The four modules or domains can be arranged in any order but typically in any of the three basic configurations of Figures 1(a), 1(b), and 1(c) of which the most suitable for a particular application can readily be determined experimentally.

The entire encoded protein is used to measure enzymatic activity (for example protein phosphorylation or dephosphorylation in living cells or organisms or in reconstituted systems in test tube conditions). Fluorescence Resonance Energy Transfer (FRET) is used as a readout of the distance and orientation between D and A. The R module is covalently modified by the enzyme whose activity is to be measured. The B module is released or binds upon enzymatic modification of R inducing a conformational change in the construct, as depicted schematically in Figure 2. This conformational change alters the relative orientation and distance between D and A which can be measured by the degree of FRET between the chromophores.

GTP exchange reactions may also be measured using the systems, for example GTP exchange by GTP exchange factors. A possible R and B domain pair is the small GTPase Ras (R) and its GDP-dissociation inhibitor (GDI) (B) as it is listed in Table 2. The respective Ras specific guanine nucleotide (GTP) exchange factor ("SOS") harbours the enzyme activity to be measured. The GDI binds Ras-GDP and is released by GDP/GTP exchange by SOS.

In a preferred embodiment, FRET is measured by fluorescence lifetime imaging of D and A simultaneously as herein disclosed and the preferred method of the present invention makes use of the FRET induced ingrowth in excited state population of A and simultaneous depopulation of the excited state D.

Using techniques according to the present invention, the explicit spectral separation of donor and acceptor fluorescence by optical filtering or other means is not necessary in order to make a determination of the FRET in the donor-acceptor system. This enables the use of D and A pairs which are spectroscopically not well resolved such as GFP mutants S65T (D) and YFP (A) as shown in Table 1:

Table 1: Examples for possible Donor (D)-Acceptor (A) pairs used in the constructs.

Donor (D) Acceptor (A)	Excitation peak (nm)	Emission peak (nm)	Fluorescent lifetime (ns)@ τ_ϕ / τ_m	Reference/ source
(D) S65T	489	511	2.57/2.59	Heim and Tsien
(A) YFP-10C	513	527	2.85/2.88	Orme *et all* Clontech
(D) EGFP	488	507	n.d	Clontech

(A) YFP-10C	513	527	2.85/2.88	Orme *et all* Clontech
(D) wt GFP*	395 (475)	508	n.d.	Heim and Tsien
(A) YFP-10C	513	527	2.85/2.88	Orme *et all*/Clontech
(D) MmGFP5	473	507	2.42/2.68	Zernicka-Goetz, *et al*
(A) YFP-10C	513	527	2.85/2.88	Orme *et all* Clontech
(D) MmGFP5	473	507	2.42/2.68	Zernicka-Goetz, *et al*
(A) YFP5	514	531	3.69/3.60	see below
(D) MmGFP5	473	507	2.42/2.68	Zernicka-Goetz, *et al*
(A) S65T	489	511	2.57/2.59	Heim and Tsien
(D) S65T	489	511	2.57/2.59	Heim and Tsien
(A) YFP5	514	531	3.69/3.60	see below
(D) EGFP	488	507	n.d.	Clontech
(A) YFP5	514	531	3.69/3.60	see below
(D) CFP*	432 (453)	476 (503)	1.32/2.23	Miyawaki *et al*
(A) S65T	489	511	2.57/2.59	Heim and Tsien
(D) CFP*	432 (453)	476 (503)	1.32/2.23	Miyawaki *et al*
(A) MmGFP5	473	507	2.42/2.68	Zernicka-Goetz, *et al*
(D) CFP*	432 (453)	476 (503)	1.32/2.23	Miyawaki *et al.*
(A) YFP-10C	513	527	2.85/2.88	Orme *et all* Clontech
(D) CFP*	432 (453)	476 (503)	1.32/2.23	Miyawaki *et al*
(A) YFP5	514	531	3.69/3.60	see below

* : numbers in parentheses are the side-peaks in excitation and emission of CFP and wtGFP.

@ Fluorescence lifetimes were obtained with a fluorescence lifetime imaging microscope (Squire and Bastiaens, *J Microsc.*,) from the phase shift (τ_ϕ) and demodulation (τ_m) of the emission light from GFPs excited at 488 nm with a frequency of 80.218 Mhz.

References for Table 1: Heim R and Tsien, R.Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* 6, 178-182; Orme, M., et al., (1996). Crystal structure of the *Aequorea victoria* green

fluorescent protein. *Science* 273, 1392-1395; Zernicka-Goetz, M., Pines, J., McLean Hunter, S., Dixon, JP., Siemering, KR., Haseloff, J., Evans, MJ. (1997). Following cell fate in the living mouse embryo. *Development* 124, 1133-1137; Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997). Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 388, 882-887.

YFP5 is described in Example 4 and in UK Patent Application No. 9817225.7 entitled "Fluorescent Protein" and the PCT Patent Application which claims priority from that patent application and which has the same filing date as this application.

To determine FRET by the method described here, for all the donor acceptor pairs listed in Table 1, a preferred optical arrangement is: a single excitation wavelength at 488nm using an argon ion laser line; the emission filter and beamsplitter from the filter set 09 obtained from Zeiss (excitation filter: bandpass BP 450-490nm is removed; dichroic beamsplitter: FT510nm; emission filter: LP520nm).

Table 1 describes most of the GFP mutants which are currently available; however, it will be appreciated that new GFPs are being made that may be useful in the practice of the invention. Only a few of them can be spectrally resolved and this presents a major restriction for the possible D/A pairs using current ratiometric methods. Thus, lack of spectral resolution between available GFP mutants is a problem with prior art methods. The mutants which excite in the near/close UV region (blue mutants, EBFP, and GFP wt) can be spectrally resolved from the "redshifted mutants"; however, they have low extinction coefficients and emit blue light in the spectral region where cells emit substantial auto fluorescence. The redshifted mutants which cannot be spectrally resolved from each other are 10 to 100 fold brighter than the blueshifted mutants.

Thus, the methods of the invention have the advantage that spectral resolution of D and A is not critical at all to determine FRET signals as the method is based on fluorescence lifetime measurements. Therefore, presently only with the methods described herein, use of redshifted mutants as D/A pairs becomes possible. This has the advantage that the fluorescent signals emitted are many fold brighter than for D/A pairs using blue GFP mutants and consequently makes the method much more sensitive. Furthermore, use of spectrally not well-resolved GFP mutants facilitates the set-up of the detection device and the measurement procedure. Only one detection channel is required, optical filtering is simplified and light emitted from the donor and acceptor can always be acquired simultaneously.

The methods of the invention used to determine FRET have an advantage compared to ratiometric methods such as that described by Miyawake *et al* (1997), *Nature* 388, 882 in that they use almost all of the light emitted by A and part of the light emitted by D. In contrast, in ratiometric methods donor and acceptor emission have to be separated by optical filtering which results in substantial loss of signal.

Therefore, fluorescence lifetime imaging allows the use of currently available GFP mutants as D and A pairs with the highest quantum yields and extinction coefficients.

The present invention can be applied to directly measure and localise in living cells, organisms or *in vitro* the activity of an enzyme (eg protein kinases or phosphatases, GTP-exchange factors, proteases), changes in concentration of small metabolites or ions (eg cAMP, ATP, GTP, Ca^{2++},

Mg^{2++}). The constructs of Miyawaki *et al* (1997) *Nature* 388, 882-887 can be used to measure Ca^{2+}; other useful contructs are described in the Examples. As such it can be applied to drug screening assays, genetic screening or to biosensors.

The combination of highly parallel readout by fluorescence lifetime imaging and the stable expression of the respective fluorescent constructs in living cells makes the invention particularly suitable for ultra high throughput screening (UHTS) of drugs. The measurements are carried out close to physiological conditions since the invention makes use of fluorescent protein expression in living cells, and fluorescence lifetime measurements are non-invasive.

The invention can also be used in genetic screens for phenotypes resulting in changes in enzymatic activity. The construct may also comprise the two GFPs, a fixed binding domain (Bait or B) and a variable interaction domain (R). The R domains may be derived from cDNA libraries. In this way it will be possible to screen cDNA libraries for candidate proteins interacting with the B(ait). Expression of constructs in cells which are designed to be covalently modified on the R module by a specific receptor activity can be used as biosensors for the receptor ligand. In this way direct monitoring of, for example, concentrations of hormones, chemicals, toxins, pathogens, or antibodies is possible in real time in a complex biological system.

GFP-protein constructs: basic building blocks for applications in enzyme assays.

The construct comprises nucleic acid, preferably DNA, encoding four protein modules typically linked by suitable polypeptide spacers. It is ligated into suitable vectors and expressed as a single polypeptide in cells by standard transfection or transformation methods. The construct typically can have one of three basic configurations in which the order of the four protein modules is different as shown in Figure 1.

In all cases the protein expressed by the construct typically comprises building blocks with the following functions: (1) Donor variant GFP, which has an absorption spectrum blue shifted relative to the acceptor variant GFP (D), (2) Acceptor variant GFP, which has an absorption spectrum red shifted relative to the donor variant GFP (A), (3) protein recognition module or domain which is either covalently modified by an enzyme or binds small ligands which induce a conformational change in the module (R), (4) protein binding module (B) which has a different affinity for the recognition module upon covalent modification of, or binding of small ligands to, the recognition module (R).

The polypeptide spacers between the modules are designed in such a way that: (1) conformational flexibility allows interaction between the B and R modules, (2) the individual modules can retain their intended function and (3) the distance between D and A is favourable for FRET in at least one conformation of the polypeptide construct. These can readily be determined empirically.

Suitable GFP mutants which can function as D/A pairs are listed in Table 1. Examples of the recognition module are: (1) a specific polypeptide which is phosphorylated at threonine, serine or tyrosine residues by a

protein kinase, (2) a specific polypeptide which is dephosphorylated at threonine, serine or tyrosine residues by a protein phosphatase, (3) GTP binding protein in which the GDP can be converted to GTP by a protein specific exchange factor or GTP converted to GDP by a GTPase, (4) a protein domain which binds small metabolites, second messengers or ions such as cAMP, ATP, GTP, Mg^{2+}, Ca^{2+}, $Fe^{[2+ \text{ or } 3+ \text{ or both}]}$. The B module has a different binding affinity for the R module dependent on covalent modification by enzymes or binding of small ligands to R. Examples of R and B modules are given in Table 2.

Table 2: Typical examples of possible R and B modules for the in cell based fluorescent assay for enzymatic activity.

R domain	B domain	Enzyme assay	Reference
1. Phosphotyrosine-peptide	SH2	kinase	*Nature* (1995) **273**, 573-
2. Phosphotyrosine-peptide	PTB	kinase	*Nature* (1995) **378**, 584-
3. Phospho-peptide	scFv	kinase	*EMBO J* (1994) **13**, 3245-
4. Rab4	PIN	kinase	*Science* (1997) **278**, 1957-
Ras	GDI	GTP/GDP exchange	*Curr. Opin. Struct. Biol.* (1997) 7, 786-
5. PKA regulatory domain	PKA catalytic domain	cAMP	*Nature* (1992) **349**, 694-

The listed key references in Table 2 provide the information on the nature of the domains, where to obtain them and what their normal biological function is. These papers are incorporated herein by reference.

With reference to Table 2, the following systems are preferred embodiments of the invention.

1. A specific of example how to use SH2 domain-phosphotyrosine peptide interactions as kinase assays is the Jak kinase/STAT pathway. The typical arrangement of the modules is shown in Figure 11.

EGFP, YFP-10C function as a donor/acceptor pair. Any other possible D/A pair listed in Table 1 could be used.

STAT-Y: represents amino acids 695-709 (GPKGTGYIKTELISV) of STAT which is phosphorylated at Y702 by the Jak kinase.

STAT-SH2: represents amino acids 573-690 of STAT which binds tyrosine phosphorylated STAT-Y.

Protein specific SH2 domains and the specific motifs (tyrosine phosphopeptides) recognised by them are described in Songyang *et al* (1993) *Cell* 72, 767-778 and Songyang *et al* (1994) *Mol. Cell. Biol.* **14**, 2777-2785.

2. The insulin receptor substrate IRS-1 (Kahn *et al* (1993) *Recent Rrog. Horm. Res.* 48, 291-339) and SHC protein (Pelicci *et al* (1992) *Cell* **70**, 93-104) are two examples of proteins with a phospho tyrosine binding

domain (PTB) *via* which they bind to receptor tyrosine kinases with the consensus sequence NPX-pY (Pawson (1995) *Nature* 373, 573-580). Interaction depends completely on tyrosine phosphorylation. As described in Example 3, this may be exploited to measure extracellular concentrations of growth factors or to determine the autophosphorylation activity of the receptors. The R module consists of the receptor which contains the NPXY sequence which is autophosphorylated by the receptor upon ligand binding. The PTB domain of eg SHC (amino acids 1-238 of SHC) functions as the B module.

3. scFv are single chain antibodies which are constructed from antibody V genes, and cloned for display of associated heavy and light chain variable domains on the surface of filamentous bacteriophage. (Winter *et al* (1994) *Annu. Rev. Immunol.* **12**, 433-455). This allows screening of the phage display library with eg small peptides for peptide binding antibodies. To exploit this technology for the cell based fluorescent assay such phage display are screened with phosphopeptides representing consensus sequences for specific protein kinases (a list of such consensus sequences is described in Kemp and Pearson (1990) *TIBS* **15**, 342-346). Phages identified in such screens should habour the cDNAs encoding for antibodies recognising the phosphorylated consensus sequence peptide. These cDNAs are used as (B) modules in the cell based fluorescent assay. A respective (R) module would be the cDNA encoding the kinase specific consensus sequence. Phosphorylation of the R module would allow the binding of the B module and FRET could be measured.

4. Pin1 is a conserved mitotic peptidyl-prolyl isomerase. In response to their phosphorylation on serine or threonine residues during mitosis, Pin1

binds and regulates a set of proteins with a W-F-Y-pS/pT-R-R related consensus sequence (Yaffe *et al* (1997) *Science* 278, 1957-1960; www.sciencemag.org/feature/data/974519.shl). With Pin1 as the (B) module and eg QLRSPRR (consensus sequence of rab4 a Ras like GTPase which regulates endosomal function) as the (R) module this system can be used to measure kinase activity of mitotic kinases (eg cdc2, cdk2) by the cell based fluorescent assay. As inhibition of Pin1 induces mitotic arrest and apoptosis (Lu *et al* (1996) *Nature* **380**, 544; Lu and Hunter (1995) *Cell* **81**, 413) the system may also be used for the screening of Pin1 inhibitors which should have therapeutic applications in cancer therapy.

5. A more detailed description of this system is given in Example 2.

GFP-protein constructs: basic building blocks for applications in protein interaction traps.

The basic configuration of the construct in a protein interaction trap is the same as described above except that the DNA encoding the R domain is replaced by a variable cDNA derived from a library. The B(ait) domain is constant and is the molecule of interest which is interrogated for interaction partners encoded by the cDNA library. The library of constructs is expressed in cells which are screened for positive FRET signals, indicative of interactions between the B(ait) and the variable R domain. The constructs are reisolated from positive cell clones and the cDNA of the R domain amplified by standard techniques (eg PCR). The constructs can be targeted to specific cellular compartments (nucleus, endoplasmic reticulum, Golgi, peroxisomes, mitochondria, plasma membrane) by adding compartment specific targeting sequences in order to mimic physiological conditions of an interaction. This is a specific

advantage over the yeast-two-hybrid system where interactions can only be monitored in the nucleus using the transcriptional machinery of the cell. Another advantage over conventional interaction traps is that cell stimulus (eg addition of growth factor) induced interactions between B and R can be revealed by a transient modification of R (such as phosphorylation by a protein kinase).

Several targeting vectors (mammalian expression vectors) with multiple cloning sites are available from Invitrogen (Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands). Upon cloning of the cDNAs encoding the four modules (Figure 1) into these vectors the expressed polypeptide will be targeted accordingly. Vectors are available for targeting to:

Mitochondria : pCMV/myc/mito cat. no. V822-20

Nucleus: pCMV/myc/nuc cat. no. V821-20

Cytoplasm: pCMV/myc/cyto cat. no. V820-20

Endoplasmic reticulum: pCMV/MYC/ER cat. no. V823-20

Secretory pathway: pSecTag2 cat. no. V910-20 (the insert of this vector will be targeted into the lumen of the endoplasmic and reticulum and will subsequently be secreted).

Plasma membrane: pDisplay cat. no. V660-20

In addition the constructs may be targeted to peroxisomes or plasma membrane by fusing an SKL (Gould *et al* (1987) *J. Cell. Biol.* **105**, 2923) or CAAX (Casey (1994) *Curr. Opinion Cell Biol.* 6, 219) sequence respectively to the C-terminal end of the polypeptide. Engineering of such a construct may be done using standard molecular biology methods as already described above.

FRET measurement by combined donor and acceptor fluorescence lifetime imaging.

To monitor conformational changes of the constructs described above or other suitable constructs known in the art, it is preferred that FRET determinations of the methods of the invention are used which are performed by the simultaneous measurement of fluorescence lifetime changes in donor and acceptor GFP modules.

The advantages over intensity or ratiometric methods to determine FRET include the following:

(1) lifetimes are independent of probe concentration, probe geometries and trivial reabsorption processes;

(2) optical filtering is less stringent, allowing collection of almost all the emitted fluorescent light from the acceptor and part of the donor with a single excitation wavelength, using a dichroic mirror and emission long pass filter;

(3) the readout is quantitative and directly related to FRET efficiency not requiring external calibration as in ratiometric measurements;

(4) since excited state events alternative to FRET may account for donor quenching or lifetime decrease, acceptor excited state ingrowth as measured by time resolved techniques is the strongest evidence for FRET as alternative mechanisms are most unlikely.

The method described in this invention explicitly makes use of the acceptor lifetime characteristics which are a sensitive parameter of changes in FRET. Fluorescence lifetimes of the GFP modules are measured by the frequency domain fluorescence lifetime imaging (FLIM) both in the single

and multi frequency mode (Schneider & Clegg (1997) *Rev. Sci. Instr.* 68, 4107).

The reporter construct is excited with monochromatic high frequency, amplitude-modulated light, preferably in the range 1 MHz–1 GHz, at a wavelength which is favourable for donor module excitation, using suitable apparatus such as that described hereinafter with reference to figure 12.

As a consequence of the irradiation of the combined donor-acceptor system with amplitude-modulated light at this first selected wavelength, fluorescence is emitted from the donor and acceptor molecules at a different wavelength from the irradiating, modulated light. As discussed earlier, the emitted light from the donor and acceptor molecules may have substantially overlapping spectra, which will be amplitude-modulated according to the excitation modulation, the respective fluorescence lifetimes of the donor and acceptor molecules and according to the energy transfer taking place between the donor and acceptor systems. The emitted light will also be phase shifted according to the excitation phase, the respective fluorescence lifetimes of the donor and acceptor molecules and the energy transfer taking place between the donor and acceptor systems.

The fluorescence emitted by both the donor and acceptor modules is collected through a single long pass filter. Preferably, the long pass filter has a cut-off frequency which lies between the peaks of the overlapping donor and acceptor fluorescence spectra. More generally, however, the transition region of the filter is preferably positioned such that the acceptor fluorescence spectrum dominates the filter output over the donor

fluorescence spectrum. Alternatively, the transition region of the filter may be positioned such that the donor fluorescence spectrum dominates the filter output over the acceptor fluorescence spectrum

With reference to Table 1, in one preferred embodiment, the excitation spectrum has a peak at 488 nm from an argon ion laser, the donor fluorescence spectrum has a peak at 511 nm, and the acceptor fluorescence spectrum has a peak at 527 nm. The preferred filter has a cut-off at 520 nm.

The fluorescent light is detected by a detector system which can be modulated at the same high frequency as the excitation light (homodyne detection). This detection can be done in an optical imaging system such as a microscope by using a microchannel plate coupled to a CCD camera, as described below in connection with figure 12. See also Gadella *et al* (1993) *Biophys. Chem.* 48, 221 or other suitable detection systems capable of high frequency modulation. These allow parallel readout and spatial imaging of multiple fluorescence lifetimes.

With reference to figure 12, a fluorescent sample 1 is irradiated with excitation energy 2 which is intensity modulated at a certain frequency by an acousto-optic modulator 3 driven by the amplified voltage output from a frequency synthesiser 4a. Fluorescence emission 5 from the irradiated sample 1 is separated from the excitation light 2 by a dichroic mirror 12 and an emission 13 filter, and is focused onto the photocathode 6 of an image intensifier having a microchannel plate device 7. Photoelectrons generated by the light image incident upon the photocathode surface 6 are amplified by electron cascade across the microchannel plate 7, maintaining

the spatial resolution of the image. The amplified electron image 8 exiting the microchannel plate strikes a phosphor screen 9 to generate an amplified light image which can be recorded by imaging onto a CCD camera 10.

A second frequency synthesiser 4b, phase-locked to the first and with controllable phase ϕ_G, is used to modulate the gain of the image intensifier either by applying the amplified modulated voltage signal 11 across the microchannel plate 7 or, as exemplified in figure 12, at the photocathode 6. Frequency mixing of the resulting modulated gain characteristics with the fluorescence emission signal is thus performed at every pixel of the image output 8. The phosphor screen 9 of the image intensifier behaves as a low pass frequency filter and only the low difference frequency signals are observed by the CCD 10. Output of each of the pixels of the CCD 10 is fed to a computer system for analysis. Each pixel represents a separate sample which may be analysed independently, or all pixels may be globally processed as described hereinafter.

In a single frequency mode, a weighted average fluorescence lifetime is obtained in every resolvable spatial element (voxel) by determining the phase shift and the demodulation of the fluorescence emission of the sinusoidally excited sample.

Two types of fluorescence lifetimes can be obtained from single frequency FLIM. The modulation lifetime (τ_{mod}) is derived from the demodulation of the emitted fluorescent light relative to the excitation light, and the phase lifetime (τ_ϕ) is derived from the phase shift in the fluorescent light relative to the excitation light.

τ_ϕ is different from τ_{mod} in a heterogeneous sample containing different fluorophores exhibiting excited state reactions such as FRET. The values of τ_{mod} and τ_ϕ and the relation between them is explicitly dependent on FRET between the GFP modules and can, as such, be used to diagnose and quantify conformational changes in the construct as described in the detailed example below.

An exemplary construct contains EGFP as donor fluorescent moiety and YFP-10C as acceptor fluorescent moiety (Table 1). Two modules which are typically between the fluorescent protein modules will interact upon covalent modification of the R module (eg phosphorylation recognition motif for R and SH2 domain for B, see Table 2). The construct is transfected into cells and FRET is measured by frequency domain fluorescence lifetime imaging according to the exemplary method below.

The sample 1 is excited by high frequency modulated 488 nm light from an Argon laser or an Argon/Krypton laser. The light is modulated in the MHz range of the frequency domain by a suitable acousto-optic modulator 3. The experiment is typically, but not necessarily, done in a microscope. The excitation light reaches the sample via a FT505 dichroic mirror 12. Fluorescence is collected through the same dichroic mirror and through an LP520 filter 13 onto a phase sensitive detector (6, 7, 9, 10).

For these filter settings the quantum yield ratio of donor (EGFP) to acceptor (YFP) is approximately 1/3 and the ratio between initially excited donor and initially excited acceptor molecules is 3 (ratio of extinction coefficients at 488 nm for EGFP and YFP-10C). These values are typical for this choice of green fluorescent proteins.

The modulation lifetime (τ_{mod}) and phase lifetime (τ_ϕ) of the combined emitted fluorescence are measured as discussed above.

With reference to Figure 3, the donor molecule D has a ground state population of d_0 and an excited state population of d_1, and the acceptor molecule A has a ground state population of a_0 and an excited state population of a_1. The population, d_1, of the excited state is increased by a factor $\sigma_d\phi$, where σ_d is the donor molecular cross-section and ϕ is the photon flux at the excitation wavelength, and is depopulated by a factor k_d which is the rate of donor emission. Similarly, the population, a_1, of the excited state is increased by a factor $\sigma_a\phi$, where σ_a is the acceptor molecular cross-section and ϕ is the photon flux at the excitation wavelength, and is depopulated by a factor k_a which is the rate of acceptor emission. The population, a_1, of the excited state of the acceptor is also sustained by energy transfer k_t from the donor to the acceptor system, through FRET, giving rise to ingrowth in the time-resolved fluorescence as shown in Figure 4.

With reference to figure 13, a method for determining the relative state populations of a system of two conformations of molecules which exhibit differing degrees of FRET will now be described. The method is preferably implemented using a suitably programmed computer.

The differential equations describing the time dependent populations of the donor and acceptor two-level systems described above are, therefore:

$$\delta d_0[t]/\delta t = k_d.d_1[t] \qquad [1]$$

$$\delta d_1[t]/\delta t = -(k_d + k_t).d_1[t] \qquad [2]$$

$$\delta a_1[t]/\delta t = k_t.d_1[t] - k_a.a_1[t] \quad [3]$$

$$\delta a_0[t]/\delta t = k_a.a_1[t] \quad [4]$$

Solving the differential equations for this four-level system gives equation 5 as the solution where: $I_i(t)$ is the population kinetic of the four level system in conformation i; l is the ratio between quantum yields of donor and acceptor corrected for optical filtering; k_d, k_a and k_t are as defined above; and d_{10} and a_{10} are the excited state populations of donor and acceptor, respectively, at time t=0 after delta pulse excitation.

$$I_i(t) = l d_1[t] + (1-l) a_1[t] \quad [5a]$$

$$I_i(t)/d_{10} = (l + (l-1)\delta_i) e^{-(k_d + k_t)t} + (1-l)(R + \delta_i) e^{k_a t} \quad [5b]$$

where:

$\delta_i = \frac{k_{ti}}{k_d - k_a + k_{ti}}$ and $R = \frac{a_{10}}{d_{10}}$

R is the ratio of extinction coefficients of the donor and acceptor fluorophores at the excitation light wavelength. This value can be easily measured by the absorption of the donor chromophore and the acceptor chromophore at the used excitation wavelength (step 100). l, the ratio of quantum yields can be determined by measuring independently the fluorescence emission intensity of the donor alone and the acceptor alone, using the same filter combination, where the concentration of the donor and acceptor should be equal (step 100).

A typical construct can have two possible conformations: open, as shown in Figure 2a and closed as shown in Figure 2b. Thus, the total time-resolved fluorescence $I_T(t)$ is a linear combination of $I_i(t)$ for each of the two conformations i = 1, 2 weighted by the relative population of the two

conformations (f = proportion of conformation 1) within the measured volume:

$$I_T(t)/d_{10} = fI_1(t) + (1-f)I_2(t) \qquad [6a]$$

$$I_T(t)/d_{10} = f(l+(l-1)\delta_1)e^{-(k_d+k_{r1})t} + (1-f)(l+(l-1)\delta_2)e^{-(k_d+k_{r2})t} + (1-l)(R+f\delta_1+(1-f)\delta_2)e^{-k_o t} \qquad [6b]$$

The actual excitation light used is preferably modulated with a sinusoidal waveform containing at least a fundamental frequency and possibly higher harmonic components, and thus, in the preferred embodiment, the solution of the differential equations has to be substituted in the convolution of the sinusoidal excitation profile with the exponential decay functions of equation 6. For an excitation which oscillates at a single frequency, the detector output (step 112) can be described by:

$$F(t) = Q\{E_0\sum_{i=1}^{n} a_i\tau_i + E_1\sum_{i=1}^{n}\frac{a_i\tau_i}{\sqrt{(1+(\omega\tau_i)^2}}Cos(\omega t + Tan^{-1}(\omega\tau_i)) \qquad [7]$$

where n is the number of exponential components in the system (3 for the full system as described in equation 6); τ_i are the observed fluorescence lifetimes in the system; ω is the angular modulation frequency of the excitation light; a_i are the pre-exponential amplitudes; E_0 and E_1 are the DC and AC components of the excitation light, respectively; and Q is the quantum yield of the combined fluorescence and detection system. Expanding the cosine term in a sum of sines and cosines and re-arranging the equation gives:

$$F(t) = Q\{E_0\sum_{i=1}^{n} a_i\tau_i + E_1(Cos(\omega t)\sum_{i=1}^{n}\frac{a_i\tau_i}{1+(\omega\tau_i)^2} + Sin(\omega t)\sum_{i=1}^{n}-\frac{\omega a_i\tau_i^2}{1+(\omega\tau_i)^2}) \qquad [8]$$

The experimental quantities obtained from the Fourier transform (step 114) of the fluorescence data are the time independent term d and the amplitudes of the time dependent cosine (c) and sine terms (s) of equation 8 (step 116):

$$d = \sum_{i=1}^{n} a_i \tau_i \qquad [9a]$$

$$c = \sum_{i=1}^{n} \frac{a_i \tau_i}{1+(\omega\tau_i)^2} \qquad [9b]$$

$$s = \sum_{i=1}^{n} -\frac{\omega a_i \tau_i^2}{1+(\omega\tau_i)^2} \qquad [9c]$$

Apparent fluorescence lifetimes at frequency ω can then be calculated from the experimental quantities *c*, *s* and *d*. The apparent fluorescence lifetime determined from the phase shift ϕ equals:

$$\tau_\phi = -\frac{s}{\omega c} \qquad [10a]$$

and the apparent fluorescence lifetime determined from the modulation (m) equals:

$$\tau_m = \frac{\sqrt{\frac{d^2}{(s^2+c^2)} - 1}}{\omega} \qquad [10b]$$

The polypeptide construct in a single conformation where its GFPs transfer energy with rate k_t exhibits a double exponential fluorescence decay as given by equation 5b. The fluorescence lifetime of the acceptor fluorophore can be measured independently (step 102) by specific excitation at the red-edge of the absorption of the acceptor thereby excluding donor excitation. The rate k_a ($= 1/\tau_a$) can thus be determined independently by experiment.

When *c*, *s* and *d* are measured for this system at a single frequency ω, the rates k_d and k_t can be calculated when R, *l* and k_a are known. First a_1, a_2 (pre-exponential amplitudes of excitation from conformations 1 and 2) and

τ_1 (lifetimes from conformation 1) are calculated from s, c and d using equations 9 with n=2 (three equations with three unknowns):

$$a_1 = -\frac{\omega(s + c\omega/k_a)(s^2 - 2cd + (d + s\omega/k_a)^2 + c^2(1 + \omega^2/k_c^2))}{(c - d - s\omega/k_a)(d - c + 2s\omega/k_a + c\omega^2/k_a^2)} \quad [11a]$$

$$a_2 = \frac{(c^2 - cd + s^2)(1 + \omega^2/k_a^2)}{1/k_a(d - c + 2s\omega/k_a + c\omega^2/k_a^2)} \quad [11b]$$

$$\tau_1 = \frac{c - d - s\omega/k_a}{\omega(s + c\omega/k_a)} \quad [11c]$$

Then k_d and k_t can be calculated by using equation 5b:

$$k_d = \frac{(l-1)a_1(1 + R - k_a R\tau_1) - a_2(1 + l(k_a\tau_1 - 2))}{(a_1 + a_2)(l-1)\tau_1} \quad [12a]$$

$$k_t = \frac{(la_2 + R(l-1)a_1)(k_a\tau_1 - 1)}{(a_1 + a_2)(l-1)\tau_1} \quad [12b]$$

which provides a measure of the FRET efficiency.

When a mixture of two conformations is present, the time-resolved fluorescence decay becomes a triple exponential (equations 6). The relative population of a conformer within a resolvable volume element of the optical system is a measure of the progression of the biochemical reaction of interest. This population can be determined by measuring c and s and d at a single frequency ω when k_d, k_t, k_a are measured individually for each conformation as outlined above (steps 102–110). First a_1, a_2 and a_3 are calculated (step 118) from s, c and d using equations 9 with $n=3$ (three equations with three unknowns):

$$a_1 = \frac{(1 + \omega^2\tau_1^2)(d - c + s\omega\tau_2 + \omega(s + c\omega\tau_2)\tau_3)}{\omega^2\tau_1(\tau_1 - \tau_2)(\tau_1 - \tau_3)} \quad [13a]$$

$$a_2 = \frac{(1 + \omega^2\tau_2^2)(d - c + s\omega\tau_1 + \omega(s + c\omega\tau_1)\tau_3)}{\omega^2\tau_2(\tau_1 - \tau_2)(\tau_2 - \tau_3)} \quad [13b]$$

$$a_3 = \frac{(1+\omega^2\tau_3^2)(d-c+s\omega\tau_1+\omega(s+c\omega\tau_1)\tau_2)}{\omega^2\tau_3(\tau_1-\tau_3)(\tau_2-\tau_3)} \quad [13c]$$

where

$$\tau_1 = \frac{1}{k_d + k_{t1}} \quad [13d]$$

$$\tau_2 = \frac{1}{k_d + k_{t2}} \quad [13e]$$

$$\tau_3 = \frac{1}{k_a} \quad [13f]$$

k_{t1} and k_{t2} are the energy transfer rates for the two conformations of the polypeptide. The relative population (f) of a conformer can be calculated (step 120) from the three pre-exponential amplitudes as given in equation 6b:

$$f = \frac{a_1(l+(1-l)R)}{(a1+a2+a3)(l-(1-l)\delta_1)} \quad [14a]$$

or

$$f = 1 - \frac{a_2(l+(1-l)R)}{(a1+a2+a3)(l-(1-l)\delta_2)} \quad [14b]$$

An example of lifetime traces in a parametric plot depicting τ_{mod} and τ_ϕ as a function of f are shown in Figure 6. The values for the parameters k_d, k_a, R and l are typical for the GFP donor acceptor pair EGFP and EYFP from Clontech. A clear diagnostic for energy transfer can be observed in this plot. The lifetimes τ_ϕ and τ_{mod} in the presence of substantial FRET efficiencies are longer than expected for the individual GFPs. Furthermore, above a certain percentage of FRET τ_ϕ is larger than τ_{mod}, a situation which does not arise under normal circumstances in the absence of acceptor excited state ingrowth. These diagnostic indicators for FRET

can be directly used in UHTS applications where a qualitative measure for the activity of an enzyme is necessary.

The FLIM system has been experimentally tested on a construct which is a sensor for caspase activity in cells. The construct consisted of EGFP and YFP-10C (Clontech) linked by a DEVD sequence which is recognised and cleaved by caspases. The full sequence was YFP-10C-GDEVDAIGGGGGGT-EGFP. In resting cells the GFPs in this construct transfer energy which can be detected by the described invention. Upon induction of apoptosis by adding Fas ligand to cells, the construct is cleaved by caspase activity with concomitant loss of the FRET signal. The result is summarised in Figure 7.

Multi-frequency determination of FRET.

In multi-frequency Fluorescence Lifetime Imaging Microscopy (mfFLIM) the actual excitation light used is modulated with a sinusoidal waveform containing at least a fundamental frequency and several higher harmonic components. The method of mfFLIM is described in UK patent application number 9817227.3, filed the same day as the UK priority application for this application.

With reference to figure 14, the phase shift and demodulation of the fluorescence emission are measured compared to the excitation light (step 200). From the phase shift and demodulation of the harmonic components the lifetime components of a mixture of interacting fluorophores can be obtained.

The parameters of the triple exponential decay (equation 6), describing the fluorescence decay of GFP mutants in a construct which transfer energy, can be fully resolved by taking data with at least three frequencies (each exponential component at least one frequency). By fitting the phase shift and demodulation of each harmonic component to the dispersion relationships the amplitudes and rate constants are obtained. Each harmonic term of the fluorescence, measured relative to the equivalent term in the excitation field, has both a phase lag $\Delta\phi_n = \Theta_n - \Theta'_n$ and demodulation $M_n = M_F / M_E = F_n E_0 / E_n F_0$ which vary as a function of the fluorescence lifetimes according to:

$$\Delta\phi_n = Tan^{-1}\left(\sum_{q=1}^{Q}\frac{\alpha_q n\omega\tau_q}{1+(n\omega\tau_q)^2} \Big/ \sum_{q=1}^{Q}\frac{\alpha_q}{1+(n\omega\tau_q)^2}\right) \qquad (15a)$$

$$M_n = \left(\left(\sum_{q=1}^{Q}\frac{\left(\alpha_q / \sum_{q=1}^{Q}\alpha_q\right)n\omega\tau_q}{1+(n\omega\tau_q)^2}\right)^2 + \left(\sum_{q=1}^{Q}\frac{\left(\alpha_q / \sum_{q=1}^{Q}\alpha_q\right)}{1+(n\omega\tau_q)^2}\right)^2\right)^{\frac{1}{2}} \qquad (15b)$$

where $\alpha_q = a_q\tau_q$ is the fractional contribution to the steady state fluorescence from the q^{th} emitting species. The dispersion relationships given by equations 15 can be fitted at multiple frequencies to resolve lifetimes and corresponding amplitudes of samples containing composite fluorescent species, step 202 (Gratton and Limkeman, (1983) *Biophys. J.* **44**, 315; Lakowicz and Maliwal, (1985) *Biophys. Chem.***21**, 61; Spencer and Weber, (1969) *Ann. NY Acad. Sci.* **158**, 361). The parameters k_d, k_a, k_{t1}, k_{t2}, l and R can then be calculated from α_q and τ_q using equation 5 (step 204).

f is calculated (step 206) from the obtained normalised amplitudes (see equations 6):

$$a_1 = f(l+(l-1)\delta_1$$

$$a_2 = (1-f)(l+(l-1)\delta_2$$

$$a_3 = (1-l)(R+f\delta_1+(1-f)\delta_2)$$

where δ_1 and δ_2 are functions of the rates k_{t1} and k_{t2} (see equation 5). From a_1, a_2 and a_3, l and f can readily be calculated, when R is known.

In the case where $k_{t2} = 0$, ie. when no FRET occurs for one of the conformations, f and l can be determined from a_1 and a_2 alone, since $\delta_2 = 0$ and δ_1 can be obtained from the three obtained fluorescence lifetimes.

The above process can be carried out simultaneously on multiple pixels of a sample.

Multiple parallel measurements of the time-resolved fluorescence kinetics of the probes in different states are obtained in mfFLIM. Global analysis of a multiple of such experiments has a clear advantage over individual analysis of the data at a single point (Beechem (1992) *Methods Enzymol.* **210**, 37). The physical relations that exist between the individual time-resolved fluorescence experiments can be exploited in global analysis by simultaneously fitting the whole data set to a model containing linked parameters.

For the time-resolved FRET model given above, a set of phase and modulation data as function of frequency ω is obtained simultaneously with differing values of the parameter f dependent on the local relative population of conformations of the fluorescent construct. This could

correspond to multiple samples in different experiments or to multiple pixels in an image.

Rather than analysing each frequency domain data set individually with the dispersion relationships given in equation 15 to recover the rates and amplitudes, the whole set of data can be analysed simultaneously, where the physical constraint is applied that the rates are invariant within the set of phase-modulation data and that only the amplitude terms vary. Also, the rates are independent of wavelength of detection. Therefore, different data sets can be acquired at different emission wavelengths and globally fitted, with the rates being invariant of wavelength. This global linking of the rates over different experiments allows the recovery with higher accuracy of the amplitudes from which the parameter f can be deduced.

Certain further embodiments of the invention will now be exemplified with reference to the following numbered examples.

Example 1: Construct for measurement of protease activity by FRET

Figure 8 is a schematic representation of a construct (cDNA) for measuring protease activity by FRET.

When the protease is inactive FRET occurs as EGFP and YFP-10C are close together on the same polypeptide. Cleavage of the polypeptide at the protease specific recognition site returns the FRET signal to zero as EGFP and YFP-10C are separated.

The cDNA is generated by several rounds of PCR amplifications followed by ligations into the pcDNA3.1 mammalian expression vector as described

previously. EGFP (donor) and YFP-10C (acceptor) may be replaced by any respective donor/acceptor pair listed in Table 1 (see above). The "spacer" sequence encodes, for example, a double gycine (Gly, Gly).

Results of example experiments with such a construct measuring the activity of caspase is described in the text above.

Example 2: Measurement of cAMP

Figure 9 is a schematic representation of a construct (cDNA) for measurement of cAMP.

cAMP-K_R is a regulatory subunit of the cAMP-dependent kinase (Lee *et al* (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3608-3612) and cAMP-K_C is a catalytic subunit of the cAMP-dependent kinase (Uhler *et al* (1986) *Proc. Natl. Acad. Sci. USA* **83**,1300-1304).

The cDNA is generated by several rounds of PCR amplifications followed by ligations into the pcDNA3.1 mammalian expression vector (see above).

In the absence of cAMP, regulatory and catalytic subunits bind to each other and a FRET signal is measured. In the presence of cAMP the catalytic subunit is displaced by cAMP and the FRET signal drops accordingly. Based on a calibration with defined cAMP concentrations *in vitro* cellular cAMP levels could be imaged quantitatively.

EGFP (donor) and YFP-10C (acceptor) could be replaced by any respective donor/acceptor pair listed in Table 1. The "spacer" sequence encodes e.g. a double gycine (Gly, Gly).

Example 3: Measurement of extracellular insulin

Figure 10 shows a possible arrangement of a polypeptide to measure extracellular insulin.

IR is the insulin receptor (Gustafson *et al* (1995) *Mol Cell Biol* **15**, 2500-2508).

R: Amino acid sequence NPEY in the IR (Y= aa 960) and acts as the recognition module in the construct.

pR: Tyrosine phosphorylated **R**

S1, S2, S3: Spacer moeities

EGFP, YFP: Two GFPs which can act as a donor/acceptor pair (see Table 1)

SHC aa 1-238: Fragment of SHC (Gustafson *et al* (1995) *Mol Cell Biol* **15**, 2500-2508) representing amino acids 1 to 238. SHC acts as the binding module (B).

A: expected conformation for the unoccupied insulin receptor.

B: expected conformation for the receptor occupied with the ligand insulin.

The construct can be engineered by standard molecular methods as already described above. Upon ligand (insulin) binding the insulin receptor becomes phosphorylated at Y-960. This causes binding of SHC *via* its phosphotyrosine binding domain (PTB, amino acids 1-238) to the insulin

receptor at Y-960 (Gustafson *et al* (1995) *Mol Cell Biol* 15, 2500-2508). In order to measure insulin in the extracellular space by FRET, the construct, schematically described above (configuration A in Figure 10), is stably expressed in cells preferentially lacking endogenous insulin receptor. Those receptor constructs occupied with insulin result in a FRET signal as SHC amino acids 1-238 will bind to the Y-960 region of the insulin receptor bringing EGFP(donor) and YFP (acceptor) in closer proximity to each other.

Related systems with SHC proteins interacting with phosphotyrosine peptides of tyrosine kinase receptors may be used similarly to measure nerve growth factor (NGF) (see eg Obermeier *et al* (1993) *J. Biol. Chem.* **268**, 22936-22966); epidermal growth factor (EGF) (see Pelicci *et al* (1992) *Cell* **70**, 93-104); platelet derived growth factor (PDGF) (see Yokote *et al* (1994) *J. Biol. Chem.* **269**, 15337-15343) and interleukin-2 (IL-2) (see Ravichandran & Bukaroff (1994) *J. Biol. Chem.* **269**, 1599-1602).

Example 4: Construction of mutant GFP and its properties

The mutant GFP which we call YFP5, which is a red-shifted mutant of MmGFP5, was generated by PCR-mediated site-directed mutagenesis of MmGFP5 (Zernicka-Goetz *et al* (1997) *Development* **124**, 1133-1137). MmGFP5 is a wtGFP mutated in V163A, S175G, I167T, F64L and S65T; the mutations V163A, S175G and I167T were introduced into wtGFP by Siemering *et al* (1996) *Current Biol.* 6, 1653-, and Zernicka-Goetz *et al* introduced the mutations F64L and S65T). This approach introduced mutations S72A and T203Y into MmGFP5 using primer pairs

ATGCGGCCGCGAATTCGCCACCATGGGTAAAGGAGAAGAACTT and CTGGGTATCTTGCGAAGCATTGTACGTACAATGCTTCGCAAGATACCCAG; and GAAAGGGCAGATTGATAGGACAGGTAATGCATTACCTGTCCTATAATCTGCCCTT TC and AAGGATCCTCTAGAAGCTTTTGTATAGTTCATCCATG. The underlined nucleotides indicate mismatches.

The fluorescent lifetimes of various GFP mutants are shown in Table 1.

References to Table 1

1. Heim & Tsien (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Bio.* **6**, 178-182.

2. Orme M *et al* (1996). Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* **273**, 1392-1395.

3. Zernicka-Goetz *et al* (1997). Following cell fate in the living mouse embryo. *Development* **124**, 1133-1137.

4. Miyawaki *et al* (1997). Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882-887.

The final PCR product was gel-purified, digested with *Eco*RI and *Xba*I and subcloned into pEFT7MCS. This vector is based on pEF-BOS (*Nucleic Acids Res.* (1990) Sep 11; **18(17)**, 5322 pEF-BOS, a powerful mammalian expression vector. Mizushima S, Nagata S). A modified version of pEF-BOS containing a Neo resistance expression cassette, pEF1-Neo, was

obtained from G. Baier, Innsbruck. The Neo expression cassette to make the vector smaller and introduced a T7 RNA polymerase promoter as well as several unique restriction enzyme sites downstream of the human EF1α promoter and the SV40 polyadenylation site.

Any other suitable vector, as described in the specification, may be used for the expression of the mutant GFP. The introduced mutations were verified by sequencing using Sequenase. The sequence of YFP5 is given in Figure 3.

The respective GFP mutants were expressed in cells by microinjection (Pepperkok *et al*, 1997 in "Microinjection and Transgenesis", eds. Cid-Arregui and Garcia-Carranca, Springer, Heidelberg, pp 145-154) of plasmids based on the vector pEFT7MCS and with inserts of the respective GFP encoding cDNAs. At 2h after microinjection cells were mounted on the FLIM microscope set-up and the respective lifetimes were determined at 37°C in living cells. Any suitable expression system or lifetime-detection system may be used.

CLAIMS

1. A method for the measurement of a degree of fluorescence resonance energy transfer taking place between a donor and acceptor system by the steps of:

irradiating a combined donor-acceptor system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules having overlapping spectra;

simultaneously determining a modulation lifetime (τ_{mod}) and a phase lifetime (τ_{ϕ}) of the combined emitted fluorescence of the donor-acceptor system; and

determining a degree of acceptor ingrowth by comparison of τ_{mod} and τ_{ϕ}.

2. A method according to claim 1 in which said irradiating excitation energy comprises a single wavelength of light.

3. A method according to claim 1 in which the receiving step includes the step of pre-filtering the fluorescence emissions with a long pass filter whose transition region is positioned such that the acceptor fluorescence spectrum dominates the filter output over the donor fluorescence spectrum.

4. A method according to claim 1 in which the receiving step includes the step of pre-filtering the fluorescence emissions with a long pass filter whose transition region is positioned such that the donor fluorescence spectrum dominates the filter output over the acceptor fluorescence spectrum.

5. A method according to claim 3 in which the long pass filter has a cut-off frequency which lies between the peaks of the overlapping donor and acceptor fluorescence spectra.

6. A method according to claim 1 in which the step of simultaneously determining a modulation lifetime and a phase lifetime of the combined emitted fluorescence of the donor–acceptor system includes the steps of:

carrying out a Fourier transform on the received fluorescence emission from the donor and acceptor system;

determining an amplitude of a time independent component, a time dependent cosine component and a time dependent sine component at one or multiple harmonic frequencies; and

computing an average value of τ_ϕ and τ_{mod} therefrom.

7. A method according to claim 6 in which the step of determining a degree of acceptor ingrowth by comparison of τ_{mod} and τ_ϕ with values obtained for donor and acceptor molecules separately.

8. A method according to claim 6 in which the step of determining a degree of acceptor ingrowth by comparison of τ_{mod} and τ_ϕ comprises determining whether FRET is taking place by determining whether τ_ϕ / $\tau_{mod} > 1$.

9. A method according to claim 6 in which the step of determining a degree of acceptor ingrowth by comparison of τ_{mod} and τ_ϕ includes the steps of:

independently predetermining a rate of acceptor emission;

independently predetermining a ratio of extinction coefficients for the donor and acceptor molecules;

independently predetermining a corrected quantum yield ratio for the donor and acceptor molecules; and

calculating the rate of donor emission and the rate of energy transfer between donor and acceptor.

10. A method for the measurement of a degree of fluorescence resonance energy transfer taking place between a donor and acceptor system by the steps of:

irradiating a combined donor-acceptor system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules having overlapping spectra;

carrying out a Fourier transform on the modulated fluorescence emissions from the donor and acceptor system;

determining an amplitude of a time independent component, a time dependent cosine component and a time dependent sine component at one or multiple harmonic frequencies; and

determining a measure of FRET efficiency from said components.

11. A method for determining the relative state populations of a biological system capable of having two states, in which one state exhibits a first degree of FRET between donor and acceptor molecules and in which a second state exhibits a second degree of FRET between donor and acceptor molecules, the method comprising the steps of:

irradiating the biological system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules of the first state and of the second state, the fluorescence emissions having overlapping spectra;

carrying out a Fourier transform on the measured fluorescence emissions having overlapping spectra;

determining an amplitude of a time independent component, a time dependent cosine component and a time dependent sine component at one or multiple harmonic frequencies; and

determining the relative population of the first state and the second state therefrom.

12. A method according to claim 11 further including the steps of:

independently predetermining a rate of acceptor emission for both the first state and the second state;

independently predetermining a ratio of extinction coefficients for the donor and acceptor molecules; and

independently predetermining a corrected quantum yield ratio for the donor and acceptor molecules.

13. A method according to claim 11 wherein the excitation energy is intensity modulated at a plurality of harmonic frequencies, and wherein the step of determining the relative population of the first state and the second state further comprises the step of fitting the phase shift and demodulation values of each harmonic frequency to the dispersion relationships to resolve lifetimes and amplitudes of donor and acceptor components.

14. A method according to claim 13 wherein the phase shifts and demodulation values are obtained for multiple samples having invariant

rates over the samples by linking the rates by global fitting to the dispersion relationships.

15. A method according to claim 9 in which one of the first or second degrees of FRET is nil or negligible.

16. A method of detecting a change in a biological system from a first state to a second state wherein the biological system comprises a donor and acceptor system wherein in changing from the first state to the second state a change in the degree of fluorescence resonance energy transfer takes place between the donor and acceptor system, the method comprising the steps of:

irradiating the biological system with a beam of intensity modulated excitation energy of a first wavelength;

receiving fluorescence emissions from the donor and acceptor molecules having overlapping spectra;

simultaneously determining a modulation lifetime (τ_{mod}) and a phase lifetime (τ_{ϕ}) of the combined emitted fluorescence of the donor-acceptor system; and

determining a change in the degree of acceptor ingrowth by comparison of τ_{mod} and τ_{ϕ}.

17. A polypeptide comprising, in any order in the polypeptide chain, (1) a donor chromophore, (2) an acceptor chromophore, (3) a domain R comprising an enzyme recognition site and (4) a domain B which either (a) binds to R once the enzyme has acted on the said recognition site or (b) binds to R when the enzyme has not acted on the said recognition site but does not bind to R once the enzyme has acted on the said recognition site

and when B is bound to R, and when appropriately irradiated, there is a change in the degree of fluorescence resonance energy transfer taking place between the donor and acceptor chromophore compared to when B is not bound to R.

18. A polypeptide according to Claim 17 wherein the R domain enzyme recognition site is a site recognised by and phosphorylated by a protein kinase.

19. A polypeptide according to Claim 18 wherein the B domain is able to bind the phosphorylated R domain but is not able to bind the unphosphorylated R domain.

20. A polypeptide according to Claim 18 wherein the R domain is any one of a phosphopeptide, rab4 or a PKA regulatory domain or a suitable portion thereof.

21. A polypeptide according to Claim 19 wherein the B domain is any one of an SH2 domain, PTB domain, scFV, GDI or a PKA catalytic domain or a suitable portion thereof.

22. A library of polypeptides, the polypeptides each comprising, in any order in the polypeptide chain, (1) a donor chromophore, (2) an acceptor chromophore, (3) a domain B for which it is desired that a polypeptide binding partner is identified, and (4) a domain R which may bind to domain B and wherein when R is bound to B, and when appropriately irradiated, there is a change in the degree of fluorescence resonance energy transfer taking place between the donor and acceptor chromophore

compared to when R is not bound to B, and wherein each member of the library has the same donor chromophore, acceptor chromophore and domain B, but different members of the library have different domains R.

23. A library of polypeptides according to Claim 22 wherein each domain R is encoded by a cDNA, or a portion thereof, derived from a tissue expected to express a polypeptide which binds to domain B.

24. A library of polypeptides according to Claim 22 wherein each domain R is encoded by a randomly selected polynucleotide.

25. A polypeptide or a library of polypeptides according to any one of Claims 17 to 24 wherein the donor chromophore or acceptor chromophore or both are an intrinsically fluorescent protein.

26. A polypeptide or a library of polypeptides according to Claim 25 wherein the donor and acceptor chromophores are selected from those given in Table 1.

27. A polynucleotide encoding a polypeptide as defined in any one of Claims 17 to 26.

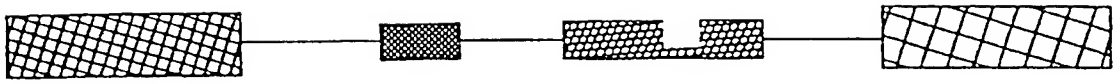
28. An expression vector encoding a polypeptide as defined in any one of Claims 17 to 26.

29. A host cell comprising a polynucleotide as defined in Claim 27 or an expression vector as defined in Claim 28.

30. Use of a polypeptide according to any one of Claims 17 to 21 or Claims 25 and 26 when dependent on Claims 17 to 21, or a polynucleotide encoding such a polypeptide, or a host cell comprising such a polynucleotide in a screening assay for a compound which modulates enzyme activity.

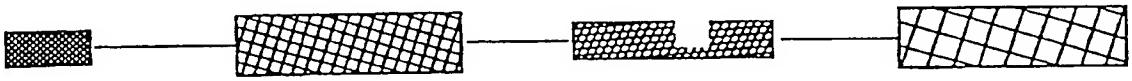
31. A method according to Claim 16 wherein the donor and acceptor system is a polypeptide as defined in any one of Claims 13 to 22.

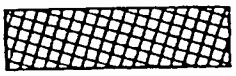
(a)

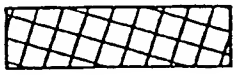


(b)

(c)



Donor (D): 

Acceptor (A): 

Binding module (B): 

Recognition module (R): 

Spacer:

Figure 1

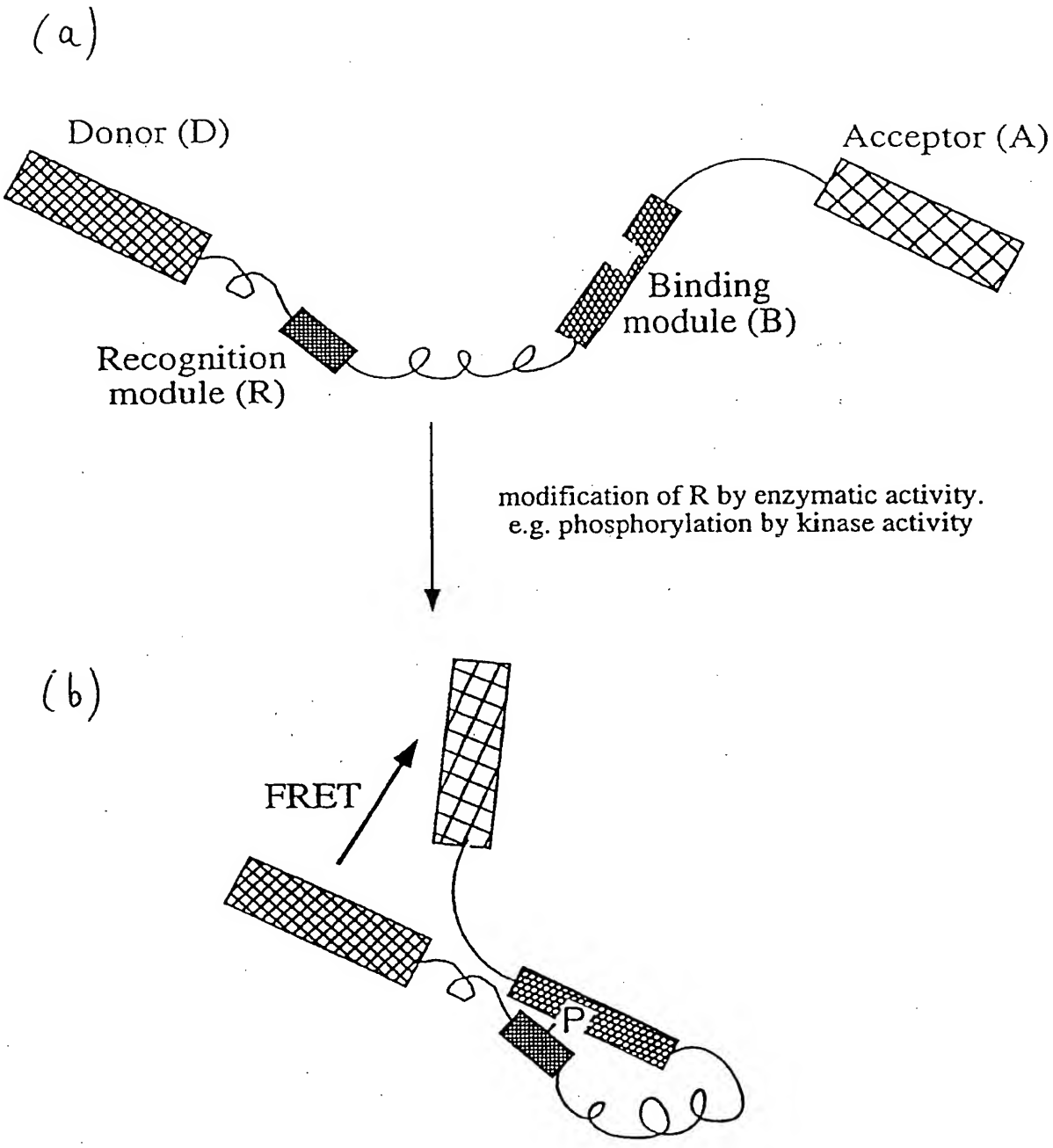
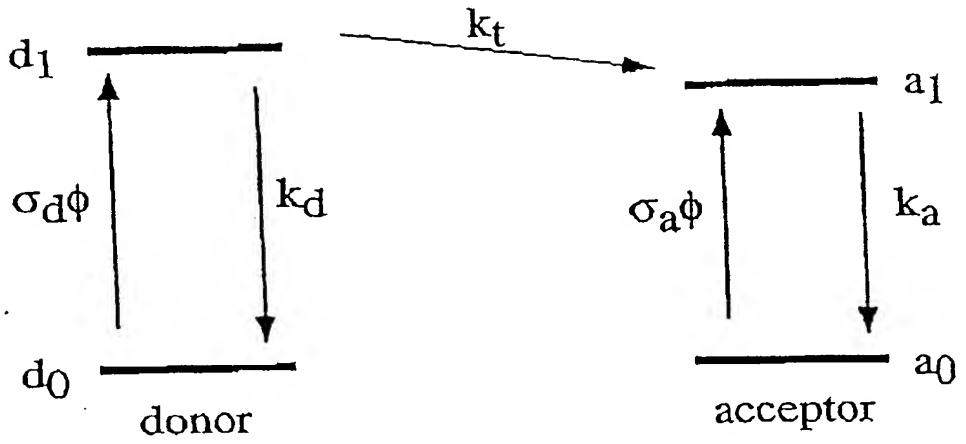

(a)
Donor (D)
Acceptor (A)
Binding module (B)
Recognition module (R)
modification of R by enzymatic activity.
e.g. phosphorylation by kinase activity
(b)
FRET
P

Figure 2

FRET between two level systems



ϕ= photon flux (ϕ) at wavelength λ
σ_d=donor molecular cross section
σ_a=acceptor molecular cross section
k_d=rate of donor emission
k_a=rate of acceptor emission
k_t=rate of energy transfer from donor to acceptor

Figure 3

Time-resolved fluorescence emission $I_T(t)$ of donor-acceptor system for different FRET efficiencies

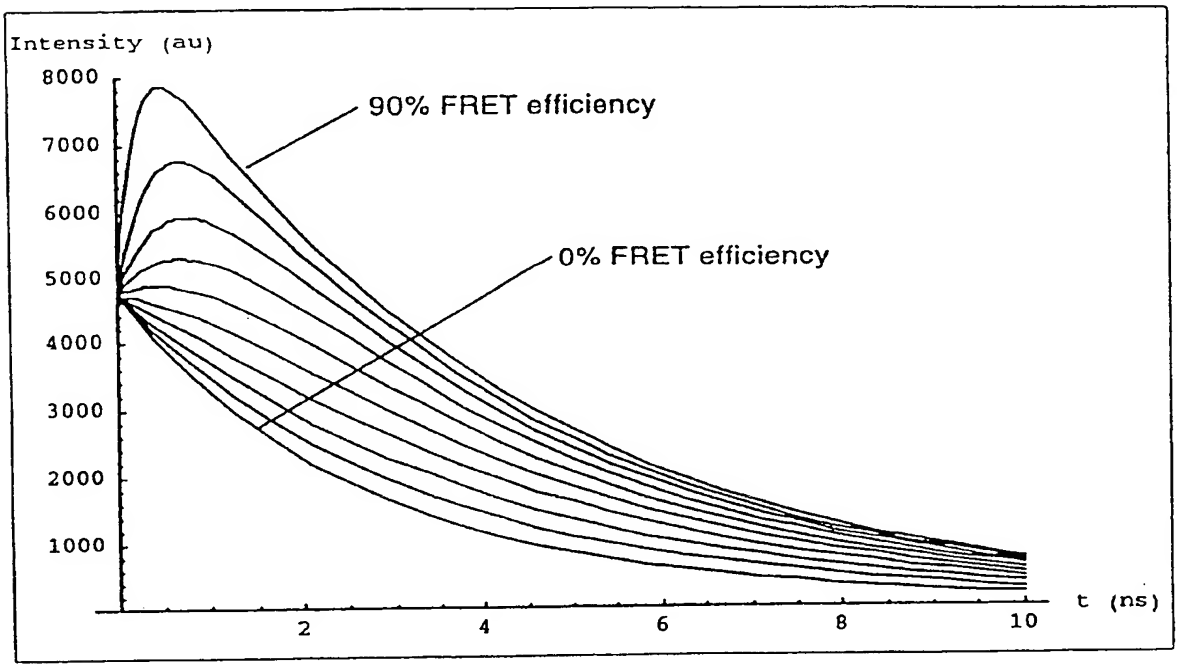


Figure 4

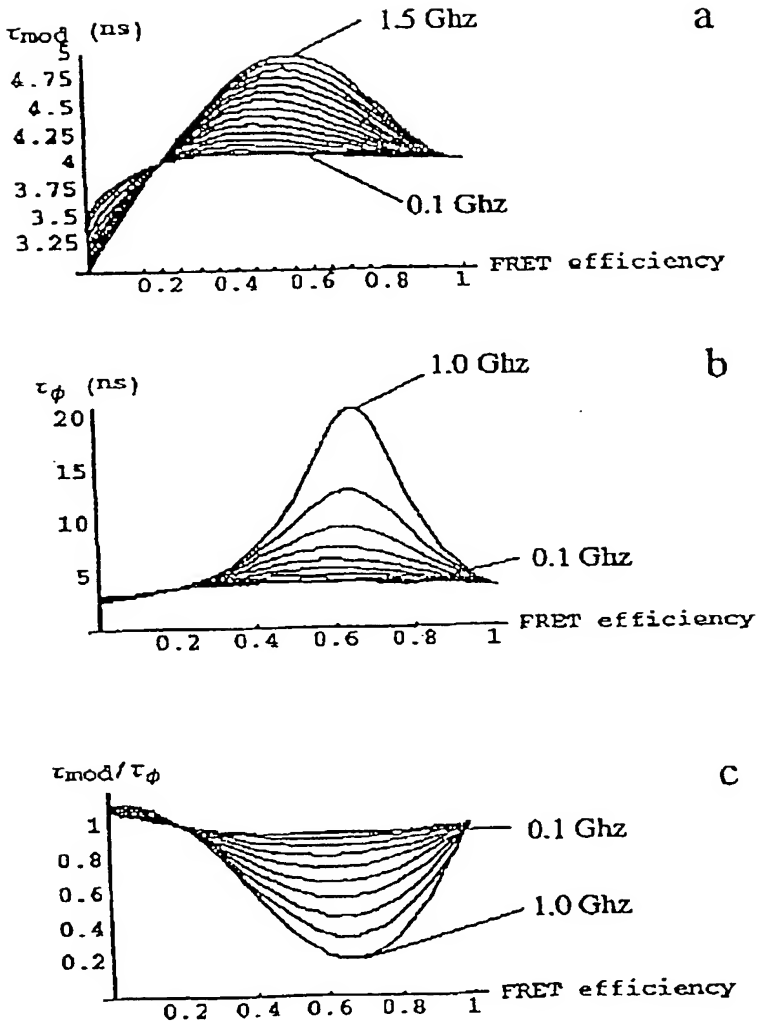

a
τ_{mod} (ns)
5
4.75
4.5
4.25
4
3.75
3.5
3.25
1.5 Ghz
0.1 Ghz
0.2 0.4 0.6 0.8 1
FRET efficiency
b
τ_ϕ (ns)
20
15
10
5
1.0 Ghz
0.1 Ghz
0.2 0.4 0.6 0.8 1
FRET efficiency
c
τ_{mod}/τ_ϕ
1
0.8
0.6
0.4
0.2
0.1 Ghz
1.0 Ghz
0.2 0.4 0.6 0.8 1
FRET efficiency

Figure 5

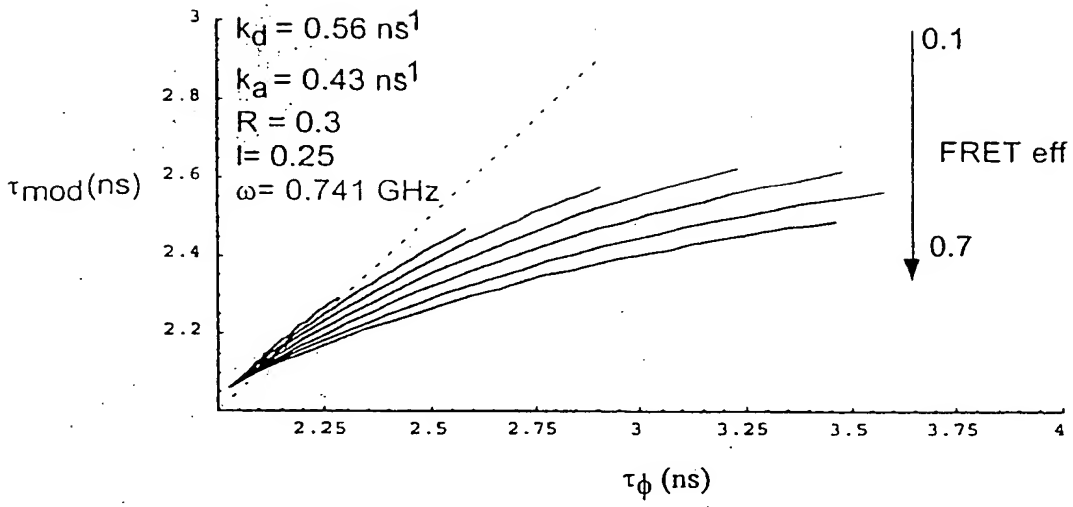

k_d = 0.56 ns^1
k_a = 0.43 ns^1
R = 0.3
l= 0.25
ω= 0.741 GHz
0.1
FRET eff
0.7
τ_{mod}(ns)
τ_ϕ (ns)
3
2.8
2.6
2.4
2.2
2.25
2.5
2.75
3
3.25
3.5
3.75
4

Figure 6

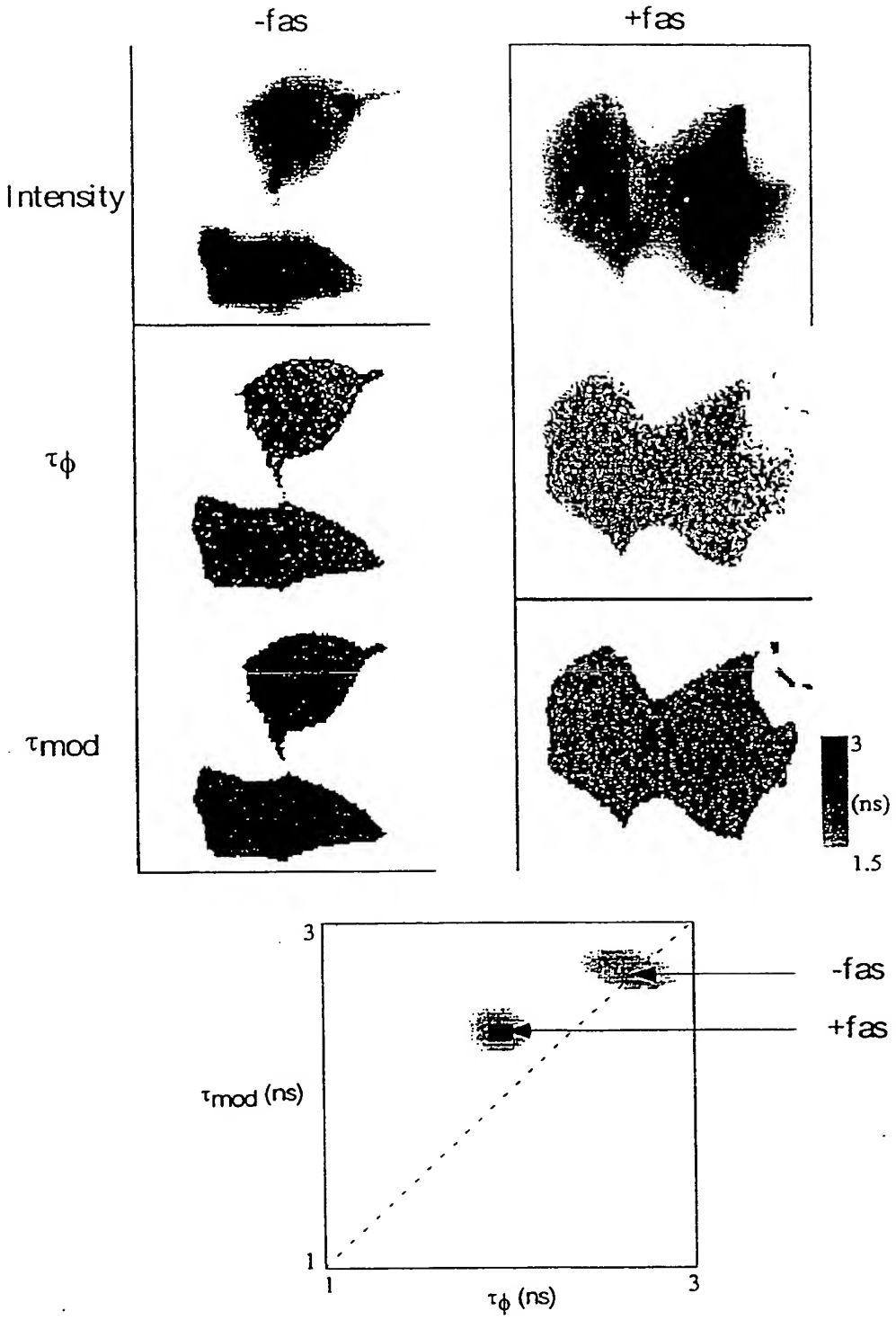


Figure 7

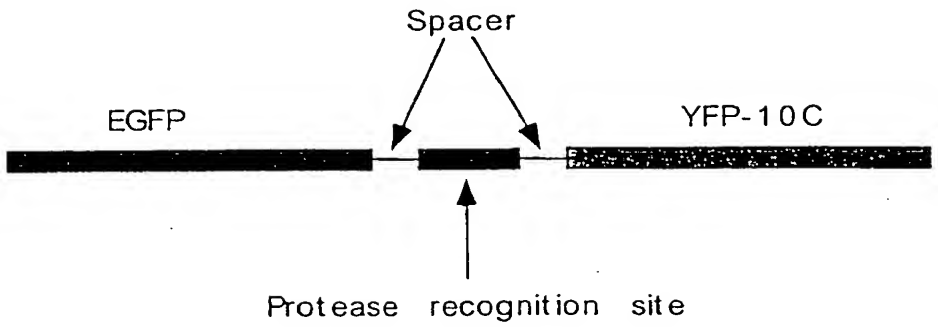


Figure 8

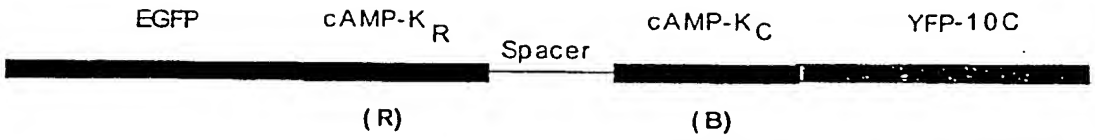


Figure 9

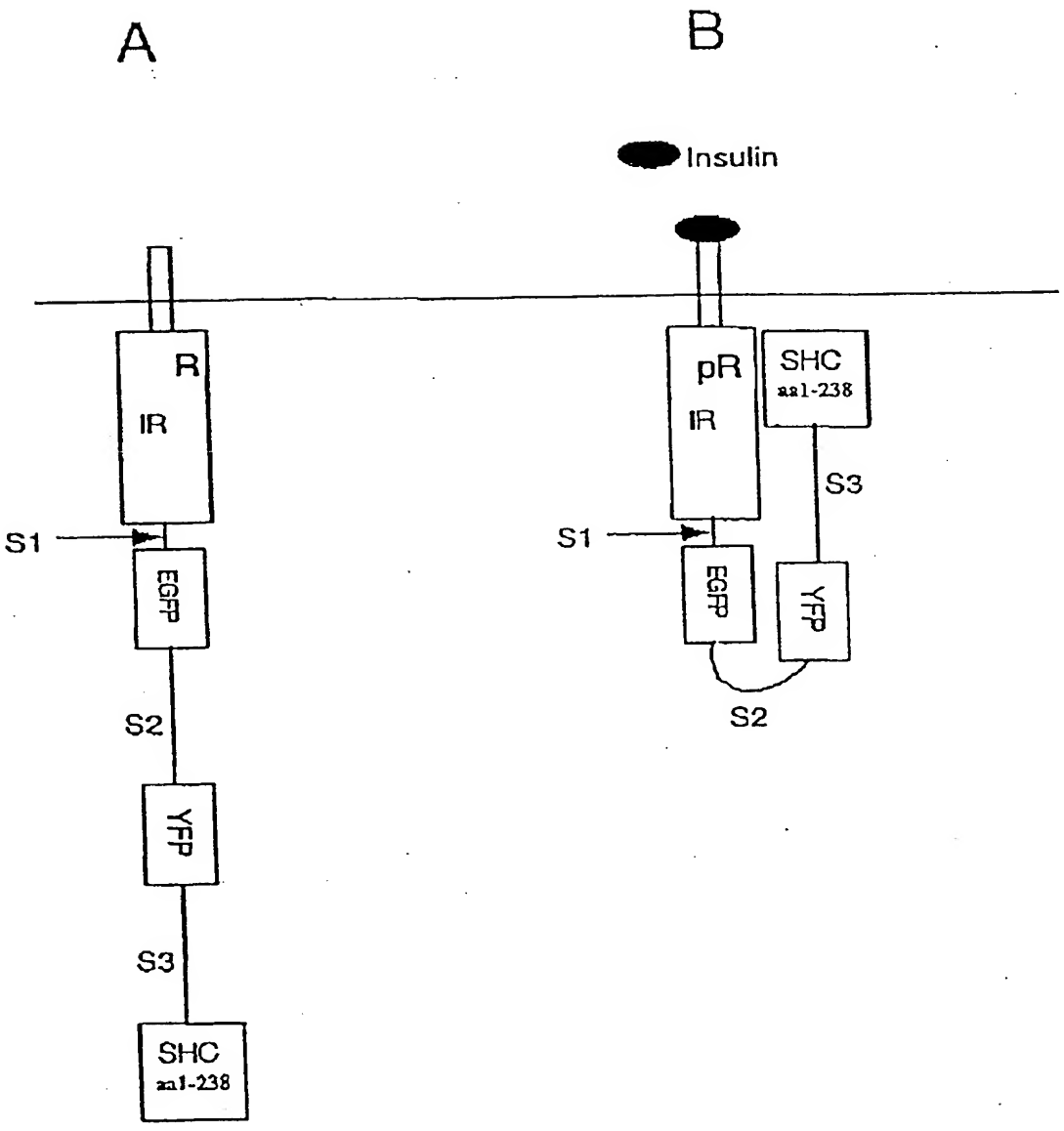

A
B
Insulin
R
IR
S1
EGFP
S2
YFP
S3
SHC
aa1-238
pR
IR
S1
EGFP
YFP
S3
SHC
aa1-238
S2

Figure 10

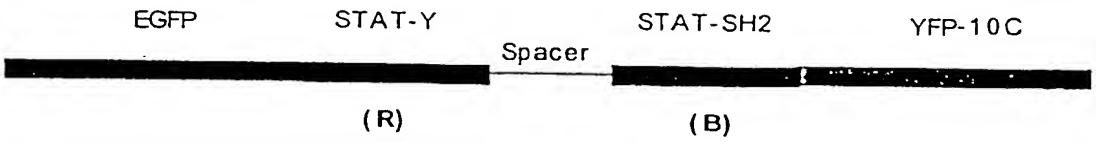


Figure 11

Fig. 12

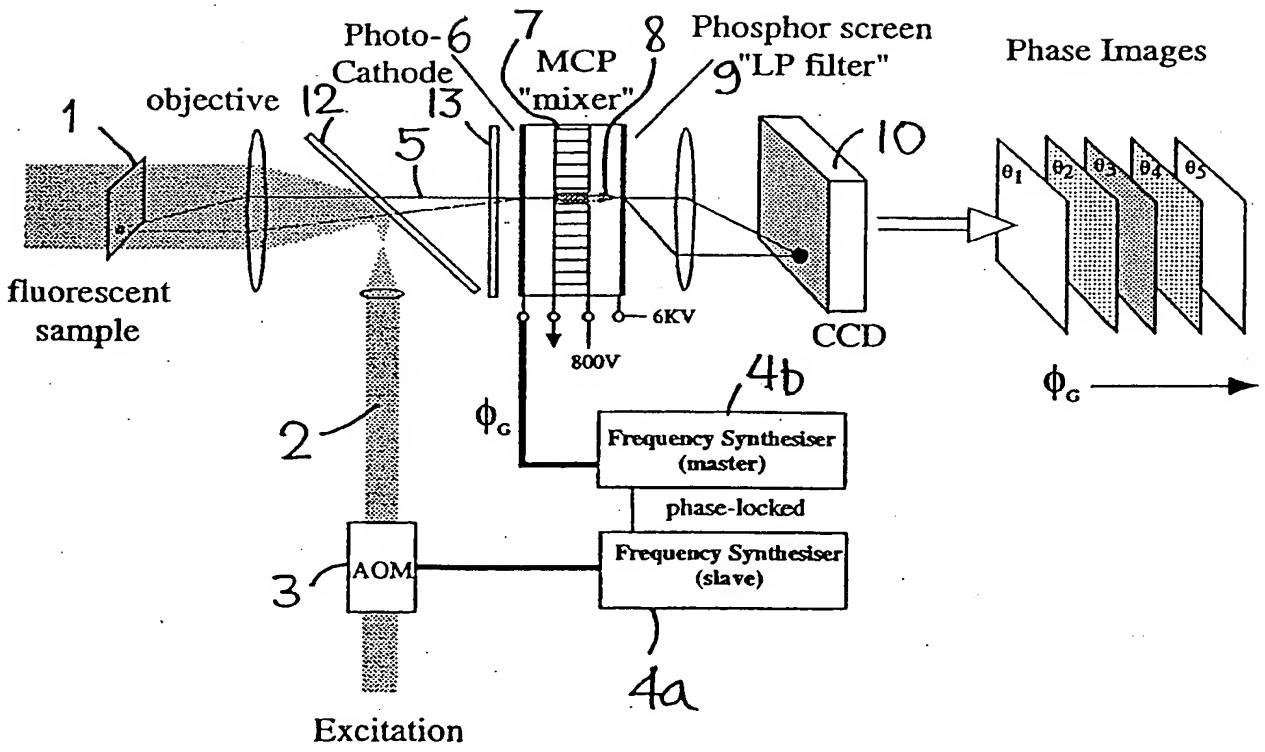


Photo-Cathode
6
7
MCP "mixer"
8
Phosphor screen
9
"LP filter"
Phase Images
1
objective
12
13
5
10
fluorescent sample
6KV
800V
CCD
θ_1 θ_2 θ_3 θ_4 θ_5
ϕ_G
4b
2
ϕ_G
Frequency Synthesiser (master)
phase-locked
Frequency Synthesiser (slave)
3
AOM
4a
Excitation

FIG. 13

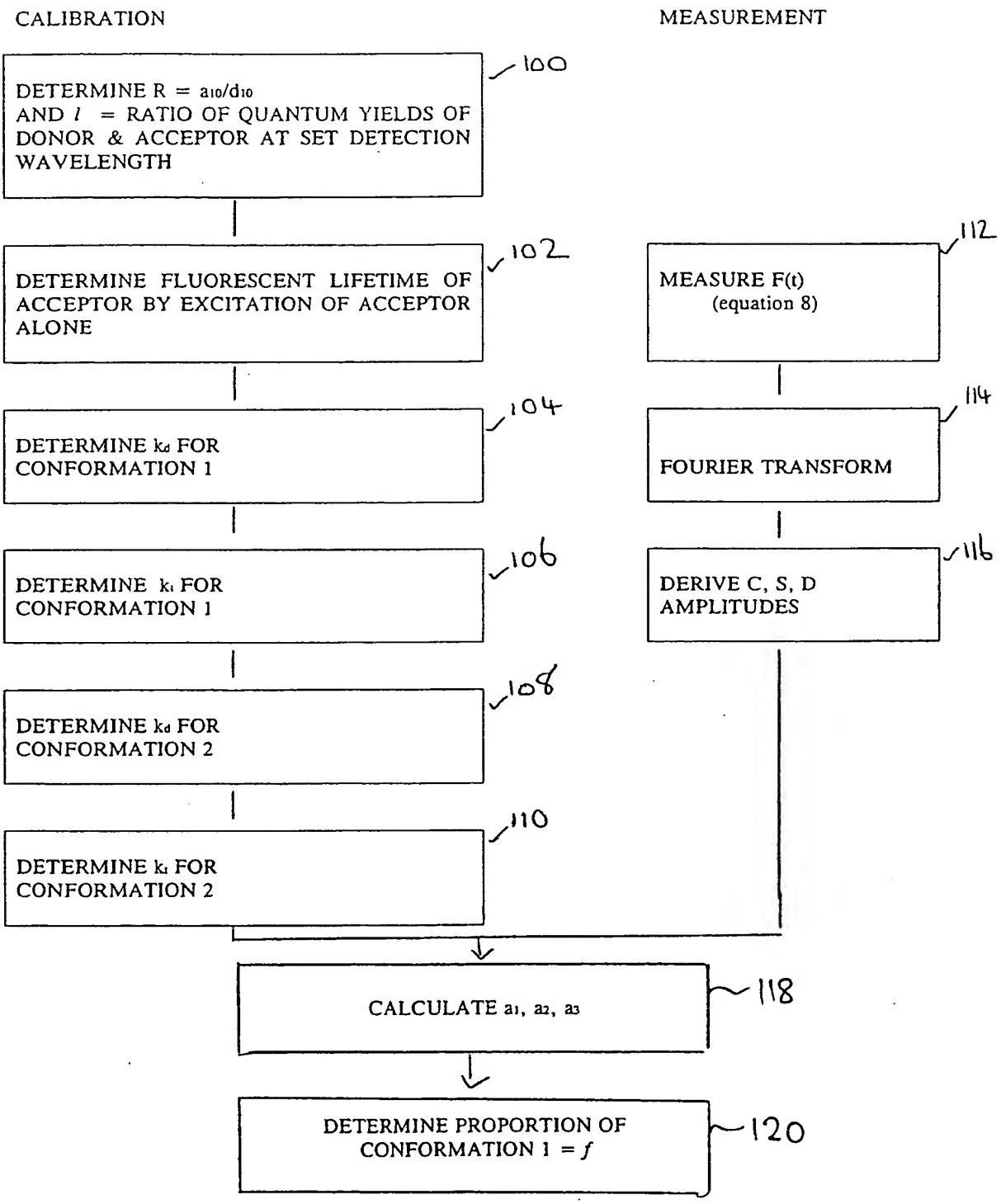
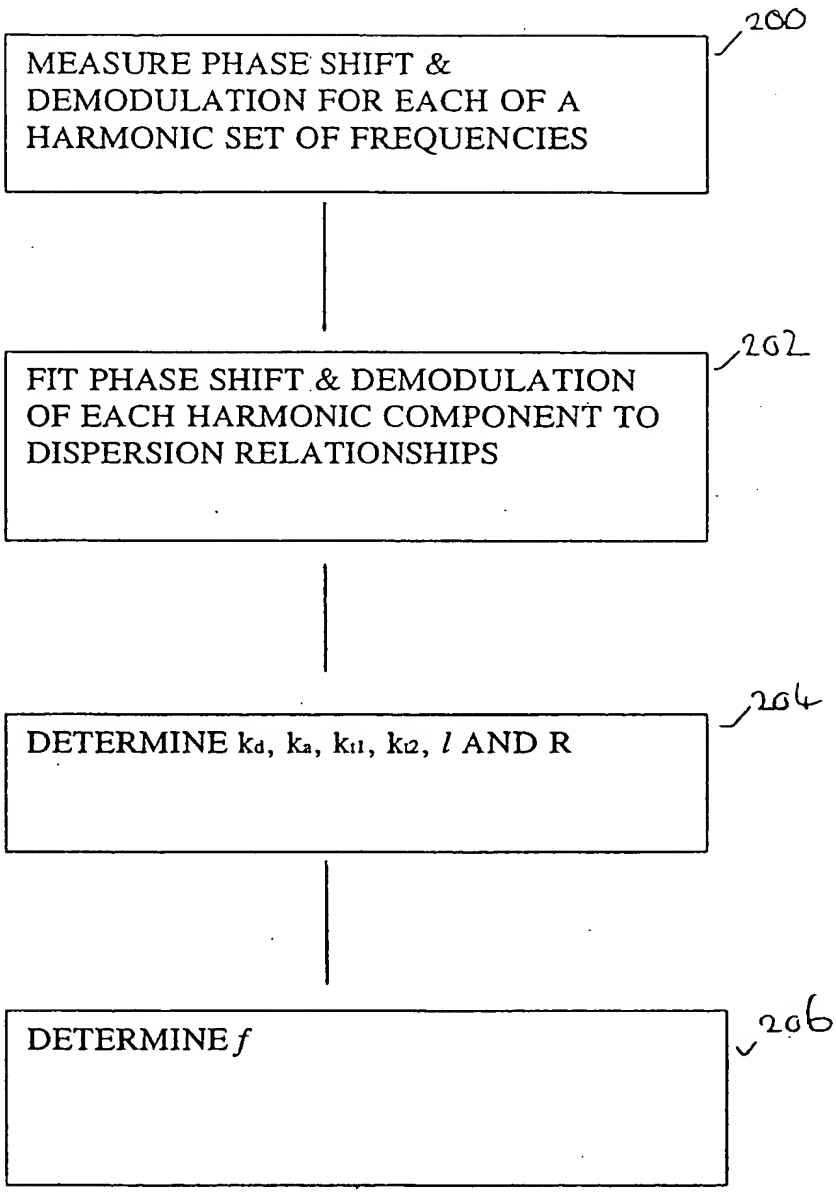

CALIBRATION
MEASUREMENT
DETERMINE R = a10/d10 AND l = RATIO OF QUANTUM YIELDS OF DONOR & ACCEPTOR AT SET DETECTION WAVELENGTH
100
DETERMINE FLUORESCENT LIFETIME OF ACCEPTOR BY EXCITATION OF ACCEPTOR ALONE
102
DETERMINE kd FOR CONFORMATION 1
104
DETERMINE k1 FOR CONFORMATION 1
106
DETERMINE kd FOR CONFORMATION 2
108
DETERMINE k1 FOR CONFORMATION 2
110
MEASURE F(t) (equation 8)
112
FOURIER TRANSFORM
114
DERIVE C, S, D AMPLITUDES
116
CALCULATE a1, a2, a3
118
DETERMINE PROPORTION OF CONFORMATION 1 = f
120

FIG. 14


200
MEASURE PHASE SHIFT & DEMODULATION FOR EACH OF A HARMONIC SET OF FREQUENCIES
202
FIT PHASE SHIFT & DEMODULATION OF EACH HARMONIC COMPONENT TO DISPERSION RELATIONSHIPS
204
DETERMINE k_d, k_a, k_{t1}, k_{t2}, l AND R
206
DETERMINE f

INTERNATIONAL SEARCH REPORT

International Application No
F ./GB 99/02598

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R.M. CLEGG: "Fluorescence resonance energy transfer" CURRENT OPINION IN BIOTECHNOLOGY., vol. 6, 1995, pages 103-110, XP002911437 LONDON, GB ISSN: 0958-1669 page 103, right-hand column, paragraph 1 page 105, left-hand column, line 10 - line 28	1,16,31
Y	page 105, right-hand column, penultimate paragraph page 107, left-hand column, line 28 - line 34	6,10,11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 1999	26/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Thomas, R.M.

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INTERNATIONAL SEARCH REPORT

International Application No
I../GB 99/02598

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	P.C. SCHNEIDER & R.M. CLEGG: "Rapid acquisition, analysis and display of fluorescence lifetime-resolved images for real-time applications" REVIEW OF SCIENTIFIC INSTRUMENTS., vol. 68, no. 11, November 1997 (1997-11), pages 4107-4119, XP002122580 AMERICAN INSTITUTE OF PHYSICS. NEW YORK., US ISSN: 0034-6748 cited in the application page 4108, right-hand column, line 3 -page 4109, left-hand column, line 4 page 4109, right-hand column, last paragraph -page 4110, left-hand column, line 10 page 4111, left-hand column, paragraph 1 page 4114, right-hand column, last paragraph -page 4115, right-hand column, line 3; figure 2 ---	6,10,11
X	T.W.J. GADELLA ET AL.: "Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy..." THE JOURNAL OF CELL BIOLOGY., vol. 129, no. 6, June 1995 (1995-06), pages 1543-1558, XP002065454 ROCKEFELLER UNIVERSITY PRESS., US ISSN: 0021-9525 page 1544, right-hand column, line 48 - last line page 1545, right-hand column, last paragraph -page 1548, left-hand column, line 9	1,2
A	page 1549, left-hand column, line 7 - line 17; figure 6 ---	3,4,10, 11,16
X	WO 98 30715 A (CIT) 16 July 1998 (1998-07-16) abstract page 6, line 4 - line 6 page 6, line 18 - line 25 page 7, line 7 - line 10 page 7, line 25 - line 26 page 8, line 17 - line 22 page 10, line 14 -page 11, line 12 page 11, line 18 -page 12, line 27 page 13, line 4 -page 14, line 15 page 24, line 12 - line 17 page 31, paragraph 1	17,18
Y	claims 1-4,22,23,25-28,38-42; figure 1 --- -/--	22-30

INTERNATIONAL SEARCH REPORT

International Application No
P ./GB 99/02598

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 28261 A (UNIVERSITY OF CALIFORNIA) 7 August 1997 (1997-08-07) cited in the application abstract page 3, line 6 - line 19 page 5, line 7 -page 6, line 8 page 14, line 3 - line 12 page 25, line 1 -page 27, line 5 figure 10 -----	22-30

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
P../GB 99/02598

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9830715 A	16-07-1998	AU 5090498 A	03-08-1998
WO 9728261 A	07-08-1997	AU 2250497 A	22-08-1997
		EP 0877805 A	18-11-1998